September 1957 · VOL. 16 · No. 3

Biology Library

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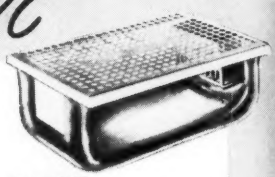
Leading Laboratories *Prefer* MOUSE-HOUSE



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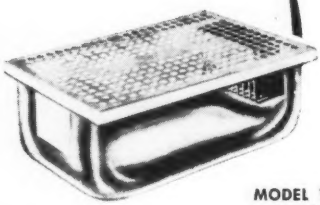

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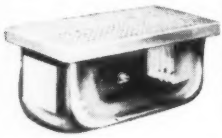

MODEL 103 STANDARD

CLEAR PLASTIC CAGE FOR MICE. This cage has all the construction features described for Model 101 except the size is smaller. Cage size for all 103 Models 11⅞" x 5⅞" x 5".

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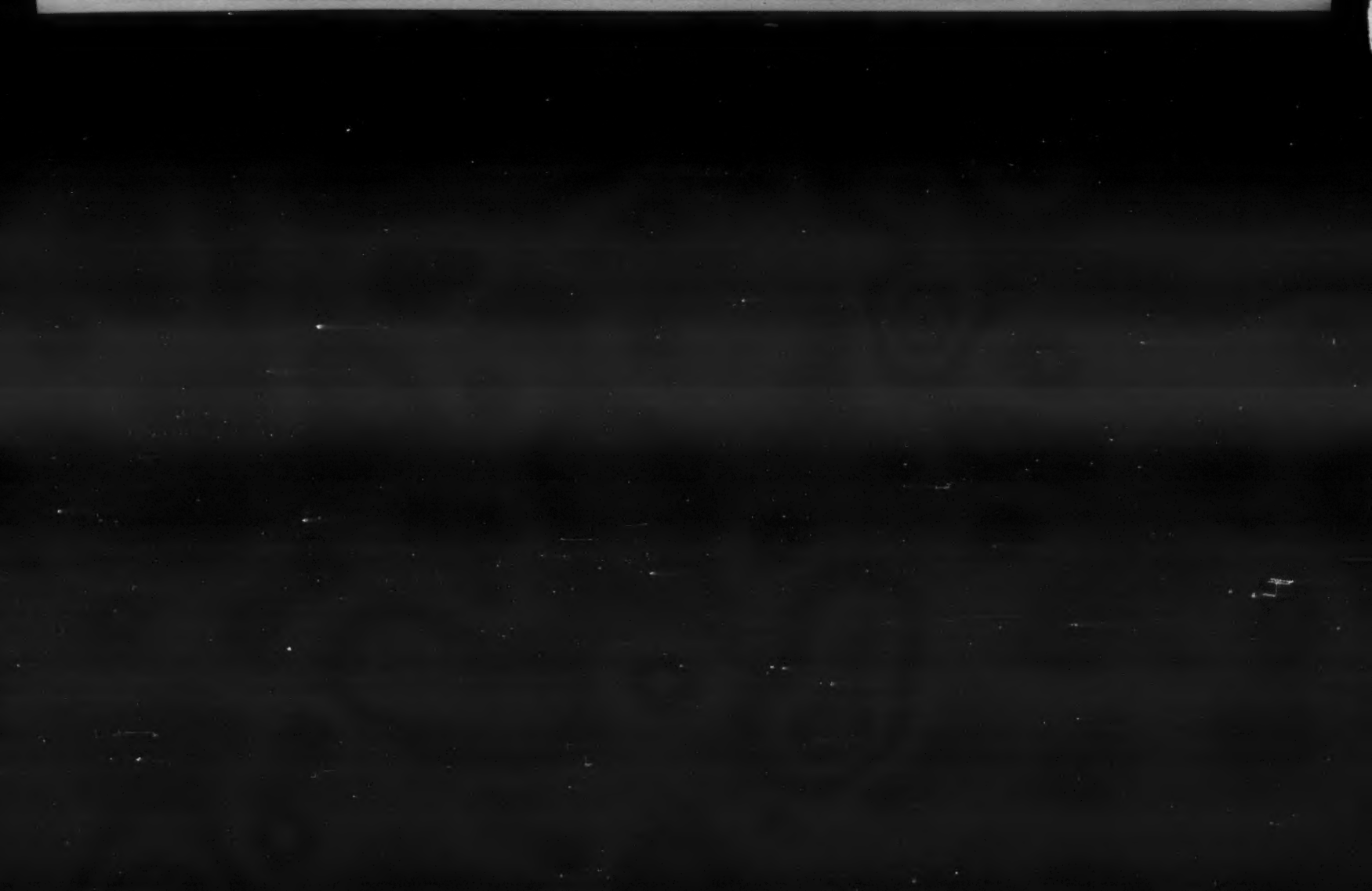

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*The American Foundation: Medical Research: A Midcentury Survey, Boston, Little, Brown and Company, 1955, vol. 1, p. XXXI.

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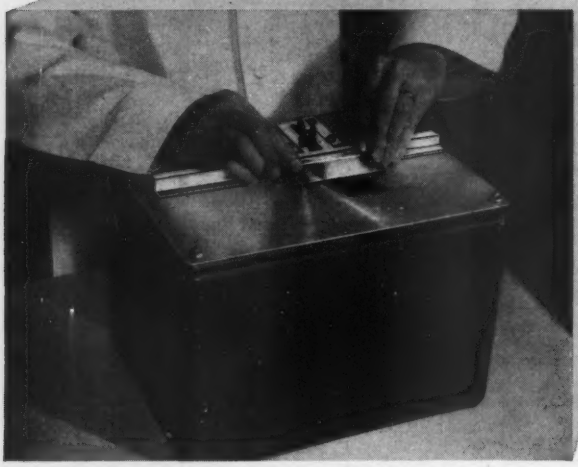

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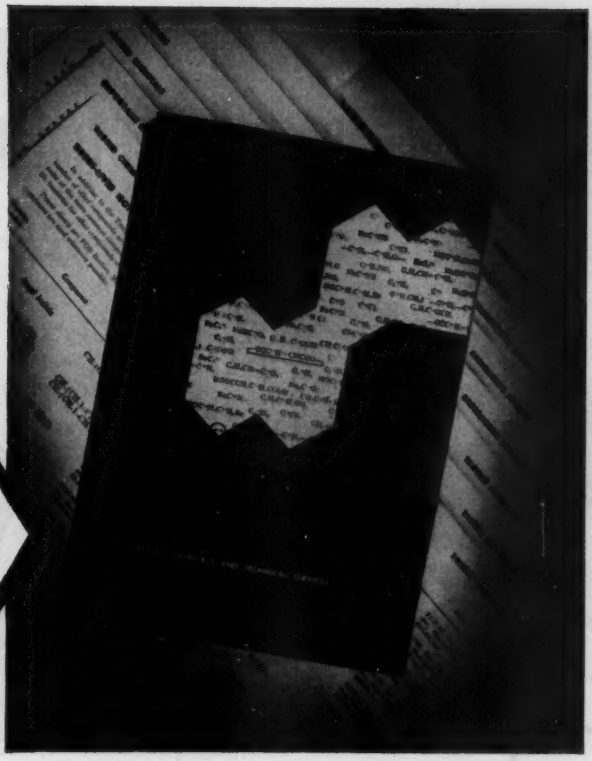

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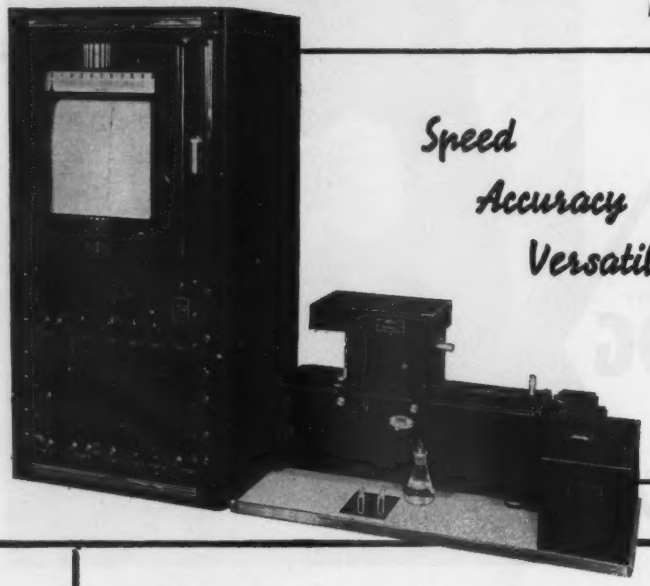

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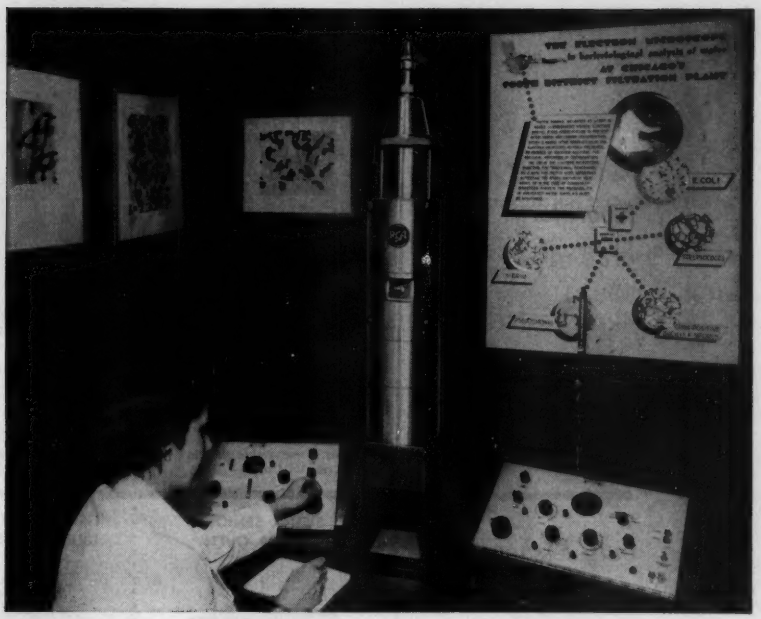

Technician at the Division of Water Purification, Bureau of Water, Chicago, Illinois, at the controls of the electron microscope.

Photomicrograph of coliform bacteria, greatly magnified.

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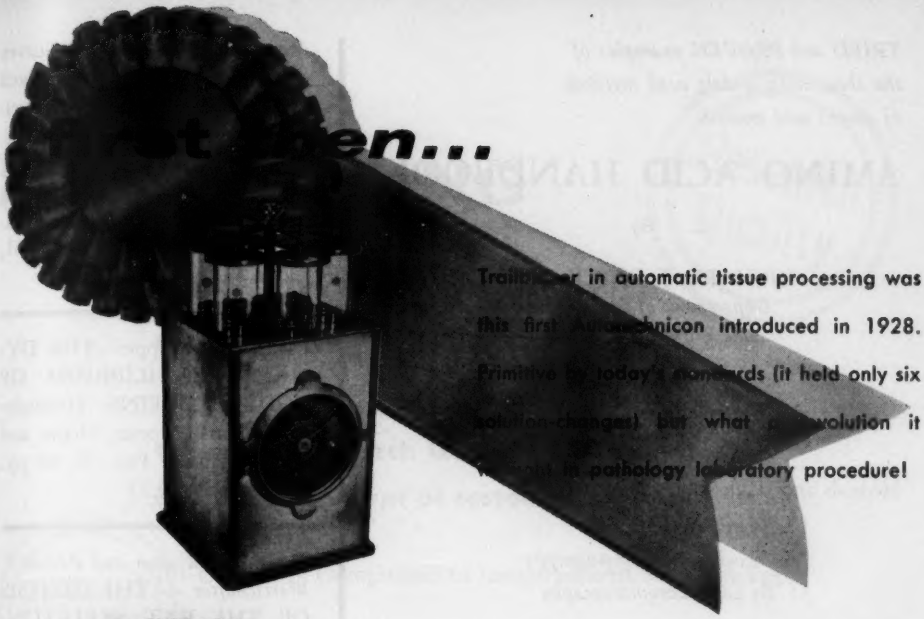

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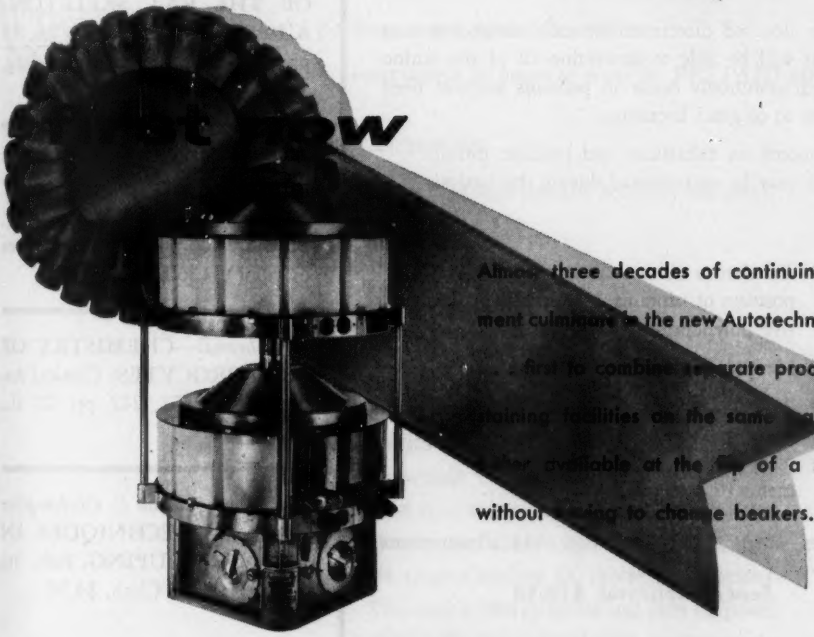

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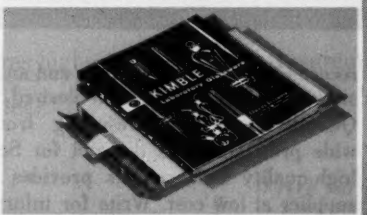

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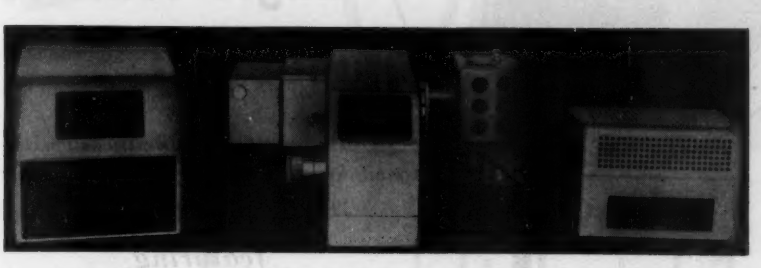

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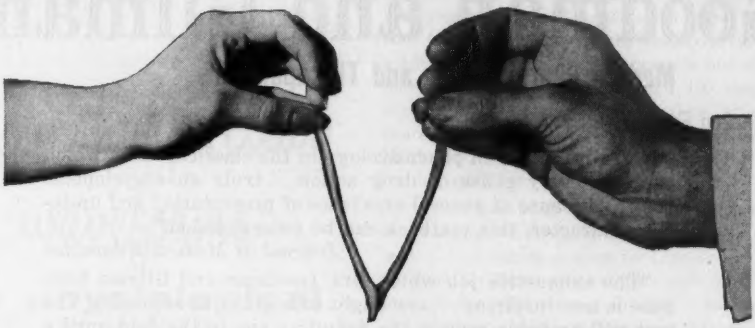

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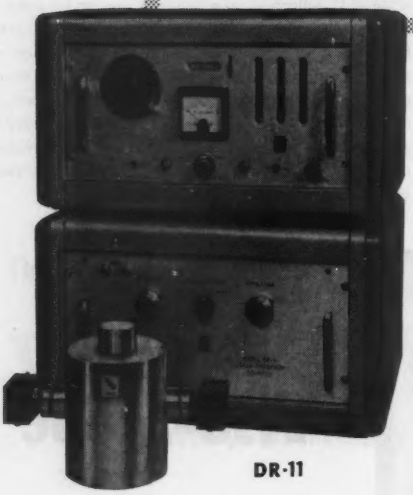

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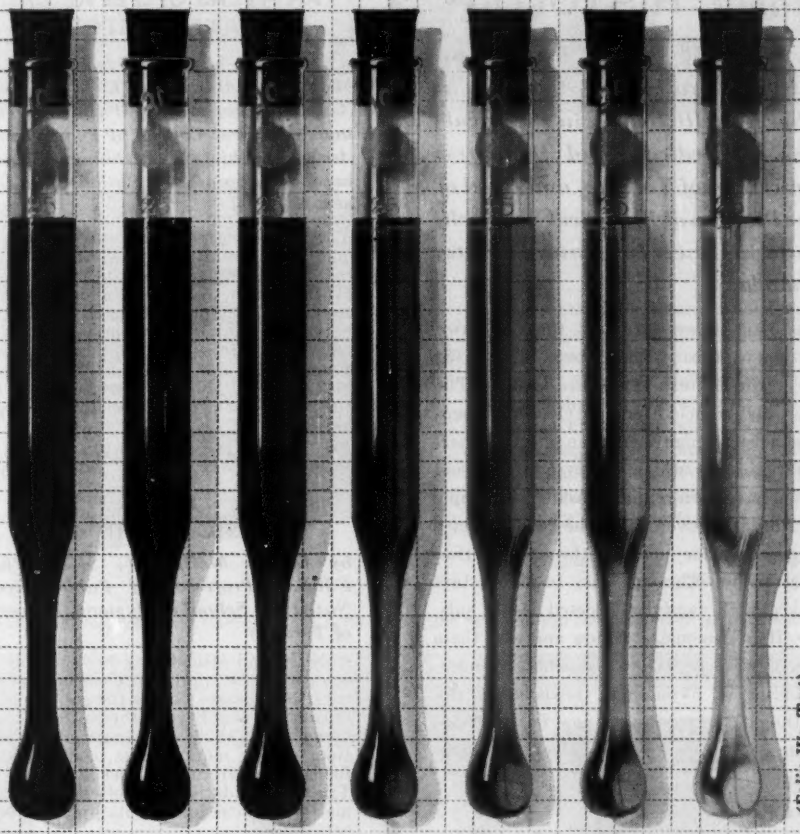

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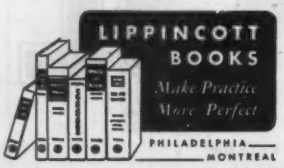

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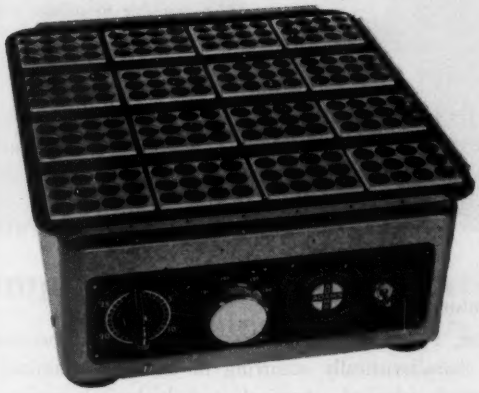

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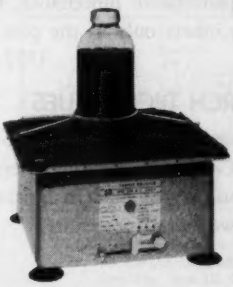

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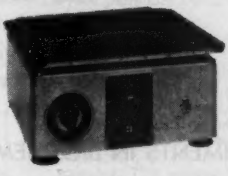

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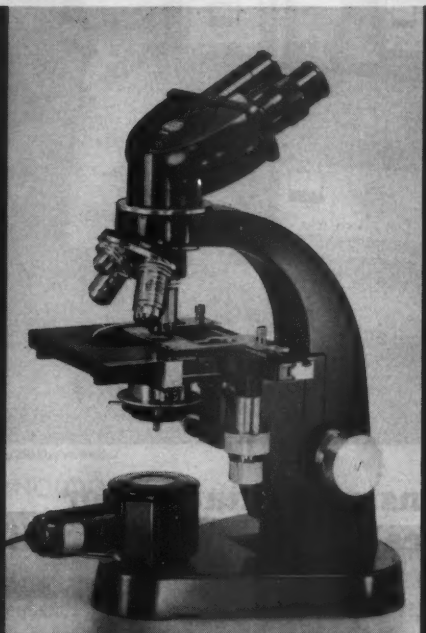

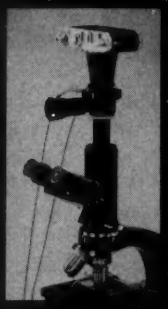

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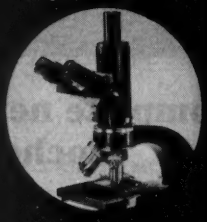

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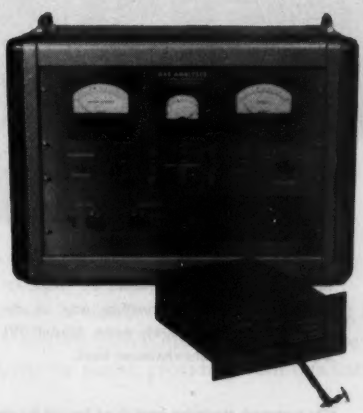

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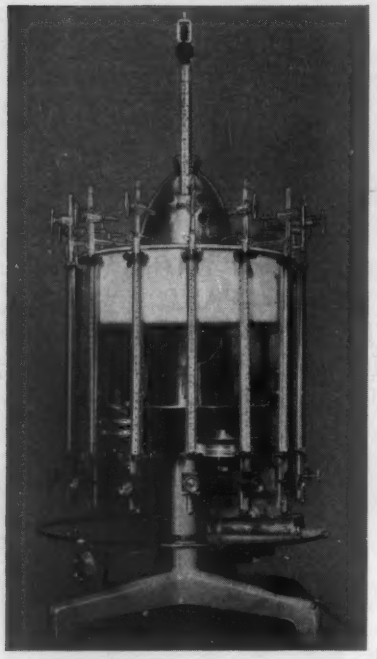

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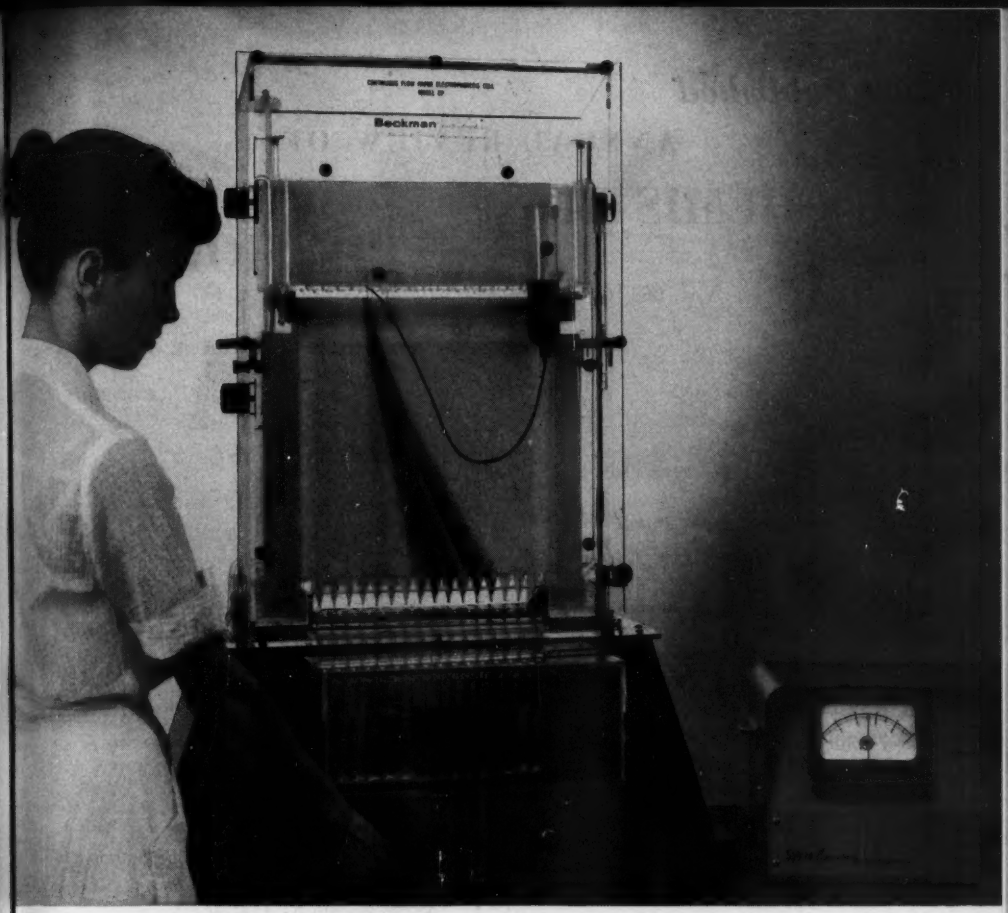

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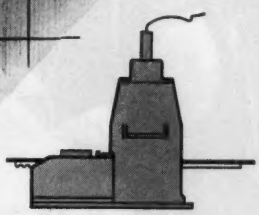

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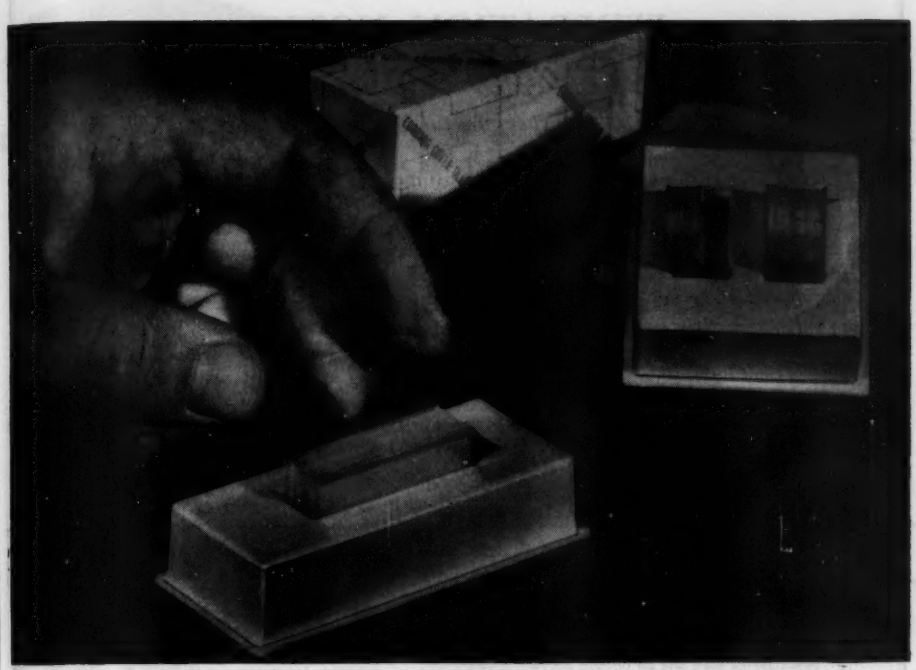

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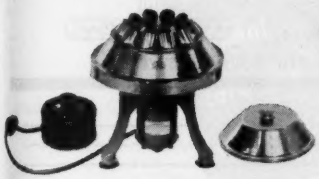

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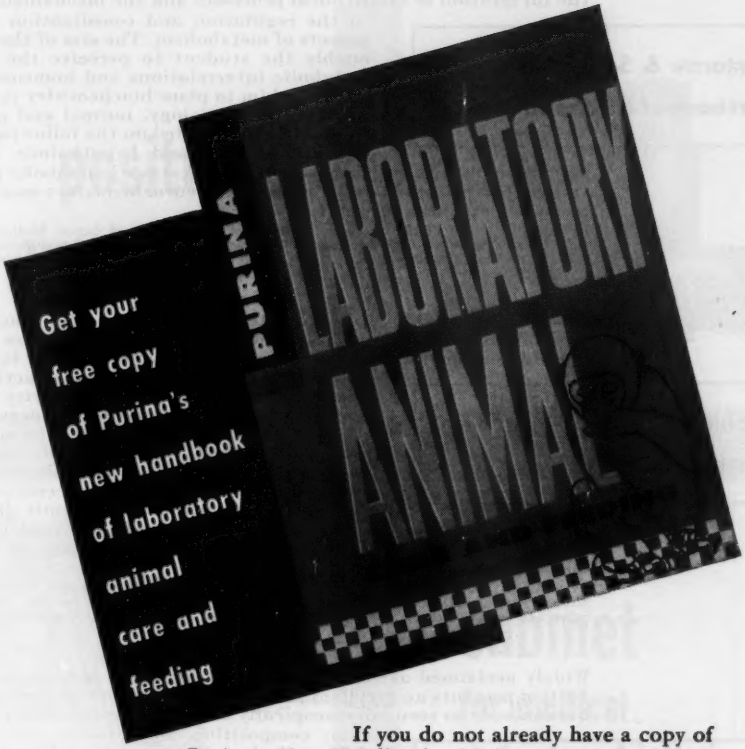

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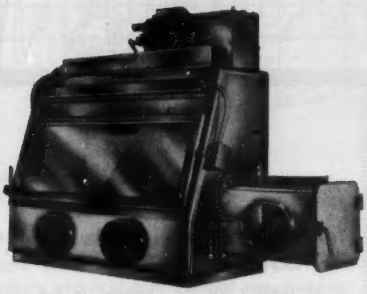

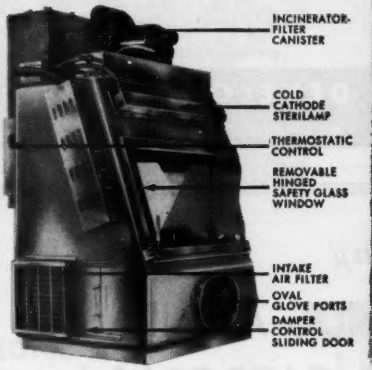

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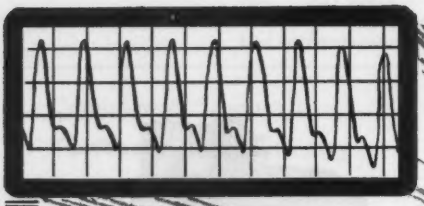

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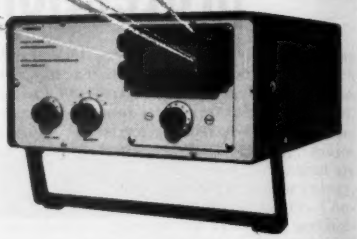

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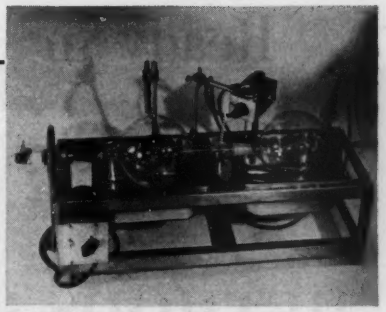

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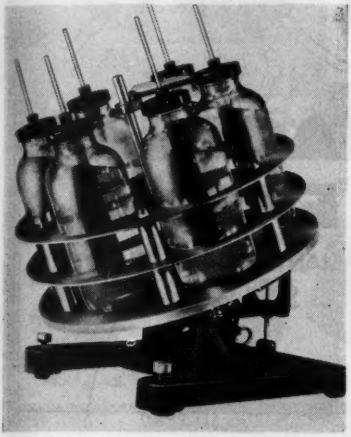

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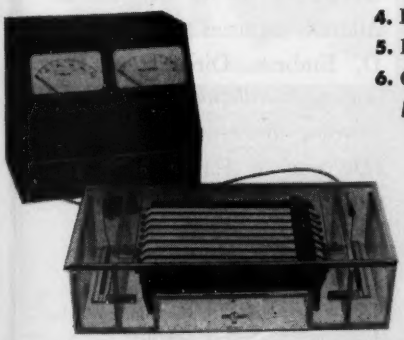

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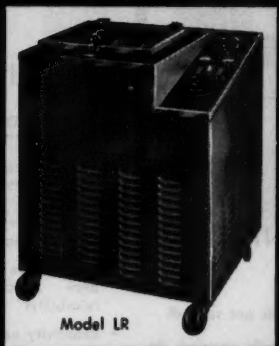

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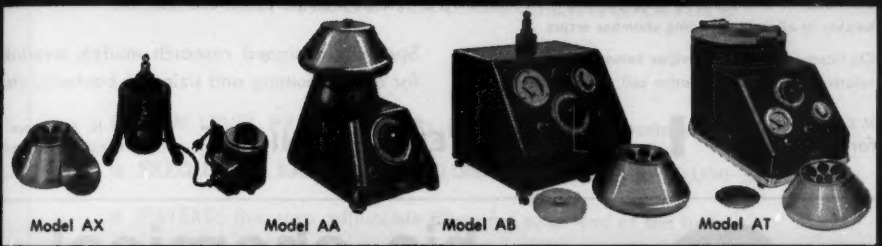

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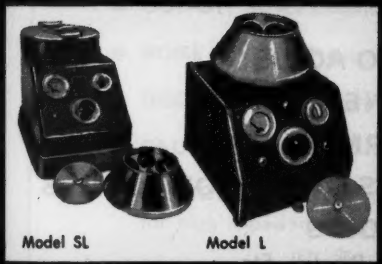

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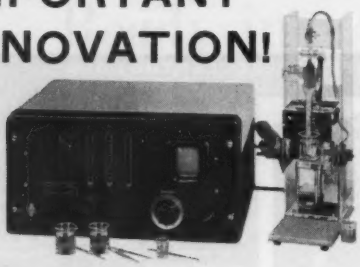

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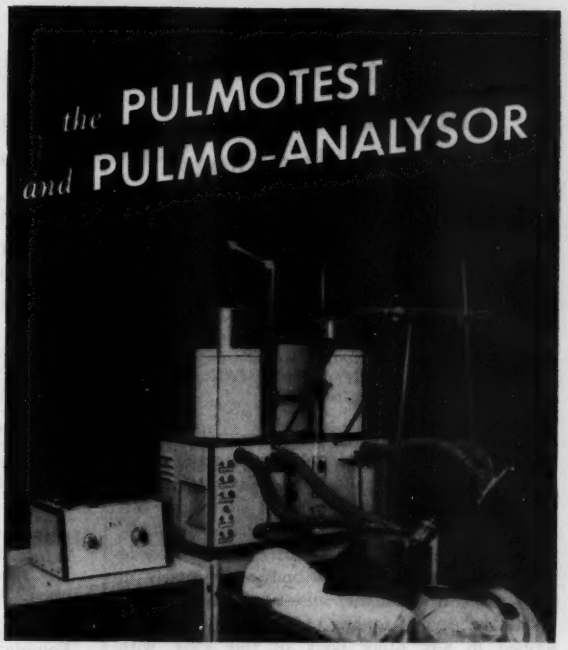

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JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. Translation of the Russian Journal. New York: Pergamon Institute, 1957. Issued monthly: annual subscription, $50.00. Published on the initiative and with the financial support of the National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare.

MEDICAL WRITING. Third Edition. Morris Fishbein, M.D. New York: Blakiston Division, McGraw-Hill Book Company, Inc., 1957. 262 pp. $7.00

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Federation Proceedings

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AAAS INDIANAPOLIS MEETING

DECEMBER 26–30, 1957

The 124th Meeting of the American Association for the Advancement of Science will include sessions of 18 AAAS sections and of some 70 participating organizations. Programs of particular interest to members of the Federation are:

I. AAAS. Presidential Address by Paul B. Sears, Dec. 28; general symposium—Moving Frontiers of Science II: Concepts That Mold Our Lives, Dec. 26 eve. and Dec. 27 aft.

II. AAAS Section C-Chemistry. Contributed papers in organic and medicinal chemistry; symposium on Pyridine Chemistry.

III. AAAS Section F-Zoological Sciences. Contributed papers in medical zoology, Dec. 27; symposium on Recent Advances in Understanding of Pituitary Function, arranged by W. R. Breneman, Dec. 27, eve; symposium, joint with Section G, on Some Unsolved Problems in Biology, Dec. 28, 29; symposium on Radiation Biology arranged by Austin M. Brues, Dec. 30.

IV. AAAS Section I-Psychology. Invited papers on Psychopharmacology, and Sensory Isolation and Deprivation, arranged by Clifford T. Morgan, Dec. 26, 28, 29.

V. AAAS Section N-Medical Sciences. Four-session symposium on The Human Integument —Normal and Abnormal, arranged by a committee, Veronica L. Conley, chairman, and cosponsored by the Society for Investigative Dermatology and the AMA Committee on Cosmetics, Dec. 28, 29, Stephen Rothman presiding; Speakers: I. The Integument as an Organ of Protection—R. B. Stoughton, R. D. Griesemer, I. H. Blank; II. Circulation and Vascular Reactions—W. B. Bean (vice presidential address), B. W. Zweifach, A. C. Burton, R. R. Kierland; III. Sebaceous Gland Secretion—E. Van Scott, A. C. Lorincz, M. B. Sulzberger; IV. Pathogenetic Factors in Premalignant Conditions and Malignancies of the Skin—A. W. Horton, H. Pinkus, F. D. Malkinson. The Theobald Smith Award, Anne Frankel Rosenthal Memorial Award for Cancer Research, and the Ida B. Gould Award for Research on Cardiovascular Problems will be presented.

VI. AAAS Section Nd-Dentistry, American Dental Association, et al. Three-session symposium on the Pharmacology and Toxicology of Fluorine, arranged by Maynard K. Hine and Joseph C. Muhler, Dec. 27, 28. Covered will be the effects of fluoride in the body, excluding dental caries, with special reference to cardiovascular and kidney diseases, mortality and morbidity rates, effect on enzymes, and effects of fluoride retention within the body.

VII. AAAS Section Np-Pharmacy, American Pharmaceutical Association, et al. Contributed papers, Dec. 27, 28, and 30; symposia on Latest Developments in Tranquilizer Field (pharmocological, chemical, clinical), Dec. 27, and on Recent Trends in Medication, Dec. 28, arranged by John E. Christian; the vice presidential address of Robert C. Anderson, Dec. 28; a tour of the Eli Lilly laboratories, Dec. 30.

SOCIETAL PROGRAMS

Alpha Epsilon Delta. Symposium: Coordination and Integration of Undergraduate and Professional Education in the Health Sciences; and Annual Luncheon and address; Tour of Indiana University Medical Center, Dec. 28.

American Association of Hospital Consultants. Symposium arranged by Jacque B. Norman.

American Physiological Society. Symposium: Recruitment and Training of Biological Scientists by High Schools and Colleges, arranged by Fred A. Hitchcock.

American Psychiatric Association. Four-session symposium on the Rehabilitation of the Mentally Ill, arranged by Milton Greenblatt, cosponsored by Section K, Dec. 29, 30.

The AAAS Annual Exposition of Science and Industry and the AAAS Science Theatre, with recent foreign and domestic films, will be prominent features of the meeting. Coupons for sleeping accommodations and advance registration will be found in SCIENCE, beginning July 19, and THE SCIENTIFIC MONTHLY, beginning August.

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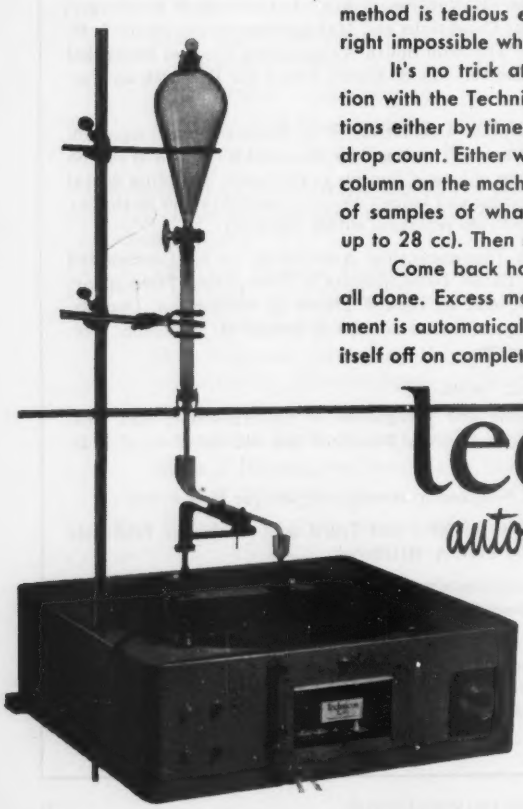

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Federation Proceedings

VOLUME 16 *September 1957* NUMBER 3

AMERICAN PHYSIOLOGICAL SOCIETY

Symposium on Tissue Oxygen Tension[1]

Chairman: JULIUS H. COMROE, JR.

INTRODUCTION

JULIUS H. COMROE, JR.

From the Department of Physiology and Pharmacology, Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

THIS symposium is one of several planned by the Americal Physiological Society to inform and stimulate biologists working in a variety of fields. The topic was selected because of its fundamental nature and broad appeal to every biologist, whether primarily a biochemist, physiologist, biophysicist, pharmacologist, general physiologist, comparative physiologist or evolutionist—whether interested primarily in the body as a whole, organ systems, organs or tissues, cells or chemical reactions and their rates—whether these organs, tissues or cells be brain, ganglia, peripheral nerve, heart, kidney, liver, muscle or skin.

This symposium consists first of broad reviews of the supply of oxygen to the blood and tissues and the adequate and optimal tissue oxygen tension for cellular function; then critical evaluation of methods, presentation of original investigative work in specific areas (kidney, brain, skin and muscle); and, finally, a panel discussion. For the last Drs. L. C. Clark, Jr., (Fels Research Institute, Antioch College), M. G. Larrabee (department of Biophysics, Johns Hopkins University), A. A. Pandazi (department of Physiology, Marquette University School of Medicine), R. H. Shepard (department of Medicine, Johns Hopkins Hospital) and R. R. Sonnenschein (department of Physiology, University of California at Los Angeles) have joined the other participants. The discussion follows the last of the formal presentations.

[1] Chicago, Ill., April 17, 1957.

DETERMINANTS OF TISSUE OXYGEN TENSION

SEYMOUR S. KETY

From the Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland

THE maintenance of an optimal oxygen tension about each cell is one of the cardinal functions of the circulatory and respiratory systems of higher animals, and an adequate explanation of the mechanisms involved in its accomplishment constitutes a large segment of physiological knowledge. August Krogh (1), in 1919, prompted perhaps by his interest in explaining biological phenomena in terms of known physical forces, made a classical and extensive study of this subject, including measurements of the oxygen diffusion in biological material and a theoretical treatment of the diffusion of oxygen from the capillary, which remains the basis of all subsequent treatments. Hill (2), in 1928, developed and generalized Krogh's concept and applied diffusion theory to problems of oxygenation of isolated nerve and muscle suspended in nutrient solutions. After that time, the problem lay relatively dormant until recently when determinants of gas exchange, especially that of inert gases, again challenged a number of investigators. The author (3) has treated some of the physiological determinants of inert gas exchange, and, more recently, Roughton (4) has derived a series of general equations for non-steady states applicable to inert gases and to oxygen as well. The recent interest in and development of methods for the precise evaluation of oxygen tension in blood and in some tissues by polarographic and other techniques which will be described subsequently, has reawakened considerable interest in the problem of tissue oxygen tension.

The factors involved in the distribution of oxygen to the tissue may conveniently be considered under two main headings—the determinants of arterial oxygen tension and the factors which operate at the tissue.

DETERMINANTS OF OXYGEN TENSION OF ARTERIAL BLOOD

Since the arterial blood is the source of tissue oxygen, it seems appropriate to begin with these factors which have also been the subject of much of the recent developments in pulmonary physiology. It is not difficult to list these determinants and to describe, partially at least, how they may operate in affecting the final oxygen tension in the blood which leaves the lung. The process of oxygen uptake at the lung conforms to a simple diffusion equation:

$$dQ/dt = -(DA/H)(p_a - \bar{p}_c) \qquad (1)$$

where dQ/dt represents the quantity of oxygen passing from the alveoli into the pulmonary blood, D represents the diffusion coefficient of oxygen in the pulmonary membranes, A and H representing the area and thickness, respectively, of the total membrane; p_a represents the mean alveolar oxygen tension and $\bar{p}_c$ represents the mean tension of oxygen in the pulmonary capillaries.

The alveolar oxygen tension depends in turn upon its partial pressure in inspired air, the magnitude and effectiveness of alveolar ventilation in which dead space, distribution, tidal volume and respiratory rate play important roles; it depends also upon total oxygen consumption which is responsible for the withdrawal of oxygen from the alveoli and the depression of its concentration below that in the inspired air. Alveolar oxygen tension is also affected to a small extent by the respiratory quotient, which, if significantly different from unity, causes some change in the volume of gas at the lungs. It should be noted that in a steady state, alveolar volume is not operative in determining alveolar oxygen tension.

The diffusion process includes diffusion of oxygen within the alveolar gas, which most workers regard as occurring practically instantaneously. The pulmonary diffusing capacity, however, depends upon the diffusion coefficient of oxygen through the pulmonary membranes,

[1] The author is indebted to Mr. Samuel Greenhouse, Chief of the Section on Applied and Mathematical Statistics, National Institute of Mental Health, for this and the succeeding mathematical developments.

which, from Krogh's work, may be assumed to be between one-third and one-half of its diffusion rate in water, and upon the total area and thickness of all of the membranes involved, which include the alveolar and capillary membranes as well as those regions of plasma and red cell which the oxygen must traverse before it is captured by the hemoglobin.

The mean pulmonary capillary oxygen tension is easier to name than to define or calculate; it represents that oxygen tension which, if it existed uniformly in the pulmonary capillary blood, would account for the total transfer of oxygen by diffusion. It represents some tension which exists between the venous and arterial ends of the pulmonary capillary, but unfortunately not a simple average. Many factors operate to influence this mean oxygen tension. Obviously, the value of the oxygen tension at the beginning of the pulmonary capillary, that in mixed venous blood, must be of importance, as well as the speed of traverse of blood through the pulmonary capillary which depends upon cardiac output and pulmonary capillary volume. Certain peculiarities of the hemoglobin-oxygen association, although indispensable to the organism, would better have been omitted from the process from the point of view of those who would express these phenomena in simple mathematical terms. Among these features should be listed the rates of the various chemical reactions involved in the combination of oxygen with hemoglobin, some of these rates being perhaps sufficiently slow as partially to limit the total rate of transfer. If so, this limitation can only be extremely small, and probably insignificant, since under normal circumstances, virtual equilibrium is reached between alveolar air and arterial blood as it emerges from the pulmonary capillary. The presence of the hemoglobin in blood, however, does tremendously increase its capacity for oxygen and significantly affects the mean capillary oxygen tension. Hemoglobin further complicates the situation by the fact that its association with oxygen does not follow any simple law and that the uptake of oxygen by whole blood is not at all described by Henry's law for the uptake of gases by liquids. Bohr (5), in 1909, derived a mathematical formula for the calculation of mean pulmonary capillary tension which could be relatively simple and explicit for an inert gas, but which, for oxygen, depends upon a rather complicated, graphical integration. The values calculated are, moreover, extremely sensitive to the value for oxygen tension in the blood as it leaves the pulmonary capillary, a measure which is not easy to obtain experimentally with a great degree of precision. Total body oxygen consumption which certainly influences mean pulmonary capillary oxygen tension, does so by affecting the mixed venous oxygen tension.

In addition to the three components of the diffusion process itself which have been discussed above, two other factors not related to diffusion operate to influence arterial blood oxygen tension. The first of these is the degree of homogeneity which exists in the pulmonary system, especially with regard to ventilation:perfusion ratios in different parts of the lung. Arterial blood emerging from the lung in the pulmonary veins represents the sum of a large number of parallel processes occurring at the alveoli. Even if equilibrium is achieved in each, if there is a large spread in the values of alveolar oxygen tension throughout the lung, there will still be a discrepancy between mean alveolar and mean arterial tension, both actual and calculated, since one is averaged on the basis of air flow and the other on the basis of blood flow and these are, of course, not the same nor necessarily comparable. Thus, in pulmonary emphysema, there may be considerable difference between alveolar air and arterial blood oxygen tension, not at all on the basis of diffusion difficulty, but rather on the basis of a lack of homogeneity in the lung. Another factor which operates outside of the diffusion process is the presence of those shunts by which venous blood which has not traversed ventilated alveoli is contributed to the mixed arterial blood. Under normal circumstances this is not a significant factor. Most investigators who have made the appropriate measurements are in agreement that, under normal physiological conditions, blood sampled from a peripheral artery deviates very slightly in oxygen tension from that in mixed alveolar air.

DETERMINANTS OF OXYGEN TENSION AT THE TISSUE

Here, as in the lung, the main process concerned in the distribution of oxygen is diffusion, except that in the tissue that process is more complicated and consists not in the transfer of oxygen across a thin membrane between two well-stirred compartments, but of diffusion into the tissue mass. The process must then be defined in

terms of certain boundary conditions, and these depend upon the choice of a suitable model. Krogh chose as his model a cylinder of tissue with a capillary passing through its longitudinal axis. Although a hexagonal prism might have been more accurate, the deviations between this and a cylinder are hardly likely to be significant. The model seems a reasonable approximation in the case of many tissues, for each capillary, no matter how tortuous, may be assumed to have a roughly cylindrical diffusion field about it throughout its length. In some tissues where the capillaries form a reticulum, more appropriate models may be chosen and the diffusion phenomenon considered on that basis, although the cylindrical model represents the simplest and most general case. In figure 1, there is presented a diagram of such a cylindrical model with the important parameters of the diffusion process indicated.

Oxygen tension (p_x) at any point (x) within a tissue would be expected to depend upon the following factors: the distance (x) from the point to the nearest capillary and also to the intercapillary distance in the region (2R), the radius of the capillary (r), the rate of oxygen consumption of the tissue (m), and the diffusion coefficient of oxygen through the capillary and the tissue (d). The operation of all of these factors is clear if we consider that in a steady state oxygen is being supplied from the capillary source and being utilized at a constant rate by the tissue. There would then be a radial gradient outward through the tissue from the capillary wall to the most remote point supplied by that capillary. The steepness of the gradient would depend not only upon the metabolic rate of the tissue, but also upon the total amount of tissue supplied by the capillary, hence, its dependence on intercapillary distance. The oxygen tension at the source (p_0) would, of course, also be an important determinant.

At Krogh's request, the Danish mathematician, Erlang, developed an important equation relating oxygen tension at a point to the factors enumerated above:

$$p_x = p_0 - \frac{m}{d}\left(\frac{R^2}{2}\ln\frac{x}{r} - \frac{x^2 - r^2}{4}\right) \qquad (2)$$

where values for pressure are in millimeters of mercury, distances in centimeters, metabolic rate in milliliters of oxygen per milliliter of tissue per minute, and the diffusion coefficient expressed as milliliters of oxygen diffusing through one square centimeter per minute under a gradient of one millimeter of mercury per centimeter. Values for the coefficient for diffusion of oxygen in muscle at 37° obtained by Krogh would give an approximate value of 2.1×10^{-8} for the diffusion coefficient expressed in such units.

The Erlang-Krogh equation above is based upon the assumption of a cylindrical model with uniform consumption of oxygen at every point and with diffusion occurring only radially with no longitudinal gradient nor gradients between cylinders. It would be a rather formidable task to derive an equation which also incorporates longitudinal gradients, and even the most sophisticated treatments of the process biologically have not attempted to do so. Evidence is adduced below that even on the basis of the simplest cylindrical models, the longitudinal gradients may be of some significance, so that it is possible that their neglect may introduce considerable error in the approximation.

The expression above is incomplete in that p_0 (representing not arterial oxygen tension but that tension which exists in the capillary at the point nearest x) has not been defined in terms of physiological or measurable concepts. We can with little difficulty list the factors upon which capillary oxygen tension depends and derive an expression relating them. If we assume that oxygen consumption of the tissue is constant throughout the length of the cylinder as well as radially, and if we assume further a constant linear velocity of the blood through the tissue, then it is immediately apparent that the oxygen content of the blood must fall linearly with time and with distance as the blood traverses the capillary from the arterial to the venous end. Thus:

$$C_a - C_o = (C_a - C_v)\,(L_o/L) \qquad (3)$$

and

$$C_o = (C_a - (m/F))\,(L_o/L) \qquad (4)$$

C_o is related to p_o through the oxygen capacity of the blood and the oxyhemoglobin dissociation curve, and from these, p_o can be calculated. Thus, *equations 2* and *4* permit, in terms, at least, of the simplifying assumptions already indicated, the calculation of oxygen tension at any point in a tissue in terms of the physiological variables of arterial oxygen tension, metabolism, blood flow, the diffusion coefficient of oxygen in tissue, the

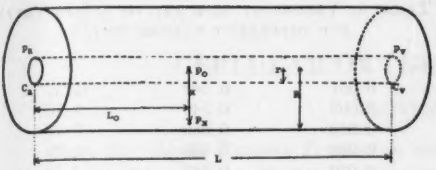

FIG. 1. A simple model for the diffusion process at the tissue.

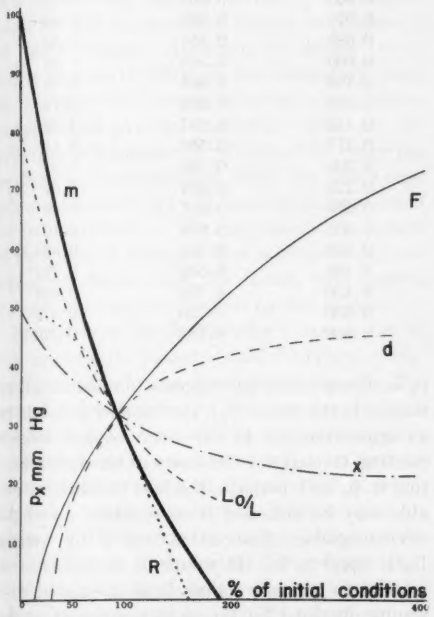

FIG. 2. Changes in Po_2 at a point within a tissue

with changes in:	from initial conditions of:
blood flow (F)	0.5 ml/ml min.
O_2 consumption (m)	0.03 ml/ml min.
diffusion coefficient (d)	2.1×10^{-5} (see text)
distance from capillary (x)	10 μ
½ intercapillary distance (R)	40 μ
distance along capillary L_o/L	0.5

(P_a is taken to be 100 mm Hg and C_a 0.20 ml/ml; C_v becomes 0.14 ml/ml)

characteristics of the hemoglobin dissociation curve, and certain geometrical dimensions of the tissue.

It would now be of interest to examine the effects of changes in each of these parameters upon the oxygen tension at a point within a typical tissue. In figure 2, such changes, calculated by the use of *Equations 1* and *4* above, are presented. For the initial conditions chosen, the tissue oxygen tension is found to be 32 mm Hg. Each of the curves represents the change in p_x with variations in one factor both above and below the initial conditions when the others remain constant. *Curve x* indicates variation in oxygen tension radially from the capillary outward in a cross-section halfway between the arterial and venous ends of the capillary. The capillary itself at that point contains blood with an oxygen tension of 50 mm Hg, which then falls asymptotically toward the point where x equals R, beyond which the tension would once more rise as the diffusion field of the adjacent capillary was entered. *Curve R* shows the dependence of tissue oxygen tension at a point on intercapillary distance. The upper limit is, of course, the oxygen tension in the blood itself at the center of the particular section, but the tension everywhere in the tissue falls very rapidly with increases in intercapillary distance. *Curve L_o/L* describes the oxygen tension at a point x distance out from the capillary as one moves parallel to the capillary from its arterial to its venous end. This is one member of a family of parallel curves, with the upper limit the change in oxygen tension within the capillary from its arterial to its venous end. *Curve d* shows the dependence of tissue oxygen tension upon the diffusion coefficient. If this were considerably reduced, the oxygen tension at many points in the tissue would be close to zero, but if diffusion rate is increased without limit, the effect would be to bring all the tissue within each cross-section to the tension level existing at the central capillary. The effect of capillary blood flow is shown by curve F. Increases in blood flow would have the same effect as shortening the longitudinal distance toward the arterial end of the capillary, so that at infinite flow the capillary blood throughout its length would contain arterial tensions of oxygen and only radial diffusion outward would be involved. The steepness of *curve m* indicates that tissue oxygen tension is highly sensitive to the metabolic rate throughout the tissue, and that is because metabolism is the only one of the factors enumerated which operates both in the process of radial diffusion and in the longitudinal gradients down the capillary. Where metabolism is zero, the oxygen tension throughout the tissue is obviously equal to arterial, but increases in metabolism above the normal level, uncompensated for by increases in blood flow or by opening up of new capillaries, would rapidly result in a deficit in oxygen tension in a large portion of the tissue.

Since the particular geometrical model chosen

may not bear a great resemblance to the actual relationships in the tissue, and because most methods for the measurement of tissue oxygen tension are not capable of measuring that function at a small point within a tissue, it would seem desirable to be able to calculate some estimate of mean tissue oxygen tension in terms of measurable physiological variables. To determine the average oxygen tension which exists throughout any section of the tissue cylinder, it is necessary to integrate *equation 2* between the limits r and R to obtain:

$$\bar{p} = p_0 + \frac{m}{d}\left[\frac{3R^2 - r^2}{8} - \frac{R^4}{2(R^2 - r^2)}\log_e\frac{R}{r}\right] \quad (5)$$

One can equate this equation[1] with the equation for oxygen tension at x (*equation 2*) to find what value of x has an oxygen tension equal to the mean tension throughout the section. After some simplification, one obtains:

$$\log_e\left(\frac{x_{\bar{p}}}{R}\right)^2 - \left(\frac{x_{\bar{p}}}{R}\right)^2 = \frac{b^2}{1 - b^2}\log_e\left(\frac{1}{b}\right)^2 - \frac{3}{2} - \frac{1}{2}b^2 \quad (6)$$

where $x_{\bar{p}}$ is the value of x at which the oxygen tension is equal to the mean for the whole section, and b is the ratio r/R. It is of interest that the radial distance from the capillary (expressed as a fraction of R), at which the oxygen tension is equal to the mean tension throughout the section, is dependent only on the ratio of the radii of capillary and tissue cylinders and is independent of metabolic rate, diffusion and capillary oxygen tension.

Table 1 lists values of $x_{\bar{p}}/R$ for different values of r/R; these were obtained from *equation 6* using Newton's method of successive approximations. More important, the last column of table 1 also offers a fairly simple means of calculating the mean oxygen tension in a particular section of tissue from the expression $\bar{p} = p_o - A\,(mR^2/4d)$ which is derived from *equation 5*. If the value of p_o is chosen which corresponds to mean capillary tension in the tissue ($\bar{p}_o$), the value of $\bar{p}$ should be an approximation to the mean oxygen tension existing throughout the tissue. One approximation to $\bar{p}_o$ and, perhaps, the best presently available may be obtained from *equation 4* and the oxyhemoglobin dissociation curve by setting L_o/L equal to 0.5. It would be of considerable interest to compare values thus calculated with results obtained by actual measurement as the newer techniques to be described become perfected.

TABLE 1. VALUES OF $x_{\bar{p}}/R$ AND OF A (SEE TEXT) FOR DIFFERENT VALUES OF r/R

r/R	$x_{\bar{p}}/R$	A
0.001	0.549	12.3
0.005	0.549	9.10
0.010	0.550	7.71
0.020	0.550	6.33
0.030	0.552	5.52
0.040	0.553	4.95
0.050	0.555	4.51
0.060	0.557	4.15
0.070	0.559	3.85
0.080	0.561	3.59
0.090	0.563	3.36
0.100	0.566	3.16
0.125	0.573	2.73
0.150	0.581	2.39
0.175	0.589	2.11
0.200	0.598	1.87
0.225	0.607	1.67
0.250	0.617	1.49
0.300	0.636	1.19
0.350	0.659	0.954
0.400	0.682	0.762
0.450	0.705	0.604
0.500	0.730	0.473
0.600	0.782	0.279

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CELLULAR OXYGEN REQUIREMENTS

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IT IS proposed here to provide a biochemical basis for the physiological oxygen requirements of tissue. Oxygen utilization by tissues occurs nearly exclusively through the respiratory chain of enzymes—the system of cytochromes, flavoproteins and pyridine nucleotides that are the principal components of mitochondria. Since the properties of cytochromes define the tissue oxygen requirements, we will describe in some detail the nature of a few of the components, the nature of the chain of enzymes that acts under physiological conditions, and the factors that control the rate of oxygen utilization by this system.

Properties of Simple Enzyme Systems. Let us now consider the measurement of oxygen utilization in a simple enzyme system (fig. 1). The general characteristics of this reaction are well illustrated by the cytochrome *c* oxidase system extracted from heart muscle and here used to illustrate the catalysis of the oxidation of ascorbic acid by molecular oxygen. Polarographic recordings of the decrease of oxygen concentration as a function of time illustrate the independence of rate from oxygen concentration until a critical concentration is reached; then the rate of oxygen utilization falls rapidly to zero. The irreversibility of the oxidase reaction assures that there is no oxygen remaining (see DISCUSSION). Half-maximal rate requires only a very low oxygen concentration. Some other enzyme systems show similar characteristics. As is indicated on the right-hand portion of the figure, peroxide utilization by peroxidase can be recorded polarographically with anodic polarization of a platinum microelectrode. This enzyme system also shows a region of constant utilization of substrate and an abrupt cessation which occurs as the substrate is expended.

Substrate-Affinity Relationships. In order to explain the enzymatic utilization of oxygen, it is necessary to define enzyme-substrate affinity. Three stages in development of these relationships are indicated in figure 2. The original Michaelis-Menten theory was based upon an equilibrium reaction of enzyme and substrate to form the intermediate. This intermediate occasionally breaks down to form free enzyme and reaction products, but the dominating reactions are the formation of the intermediate and the dissociation of substrate from the enzyme surface; the affinity constant is simply the equilibrium constant. The Briggs and Haldane modification of the Michaelis theory suggests that the breakdown of the enzyme-substrate compound is relatively more frequent than in the simple theory. Thus the affinity relationships involve the velocity constant for the breakdown of the enzyme-substrate compound.

Direct observations of the action of the enzyme-substrate compound frequently show that the affinity 'constant' is not a constant but is a function of the concentration of a second reactant entering into the enzyme process. Under such conditions the affinity 'constant' is directly proportional to the concentration of this substance. The expression for the affinity of the respiratory chain is a much more complicated one since the chain consists of a large number of enzymes operating in sequence. This expression depends not only upon reaction velocity constants but upon enzyme concentration in a very complicated manner. However, there is no unlimited increase of the affinity constant with substrate concentration, as has been indicated for the third enzyme mechanism of figure 2.

Terminal Oxidase of Respiration—Cytochrome a_3. Since the affinity of the respiratory chain for oxygen is determined largely by the nature of the reaction of oxygen and the terminal oxidase, some properties of this enzyme are presented. Much of our knowledge of the terminal oxidase depends upon its reaction with carbon monoxide, the inhibitor of respiration that competes with oxygen for the iron atom of the oxidase. The inhibition of cellular respiration by carbon monoxide was found by Warburg (1) to be light-sensitive, and a recent recording of this effect is illustrated by figure 3. In this case a platinum electrode is inserted into a drop of cell suspension

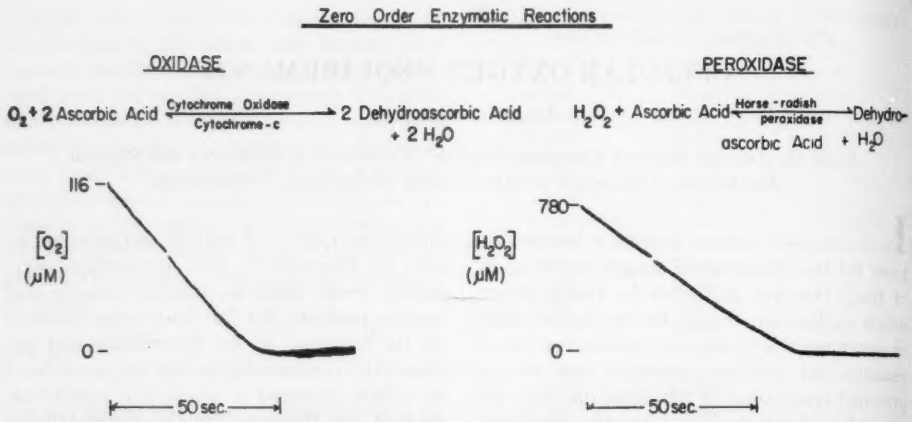

FIG. 1. Kinetics of O_2 utilization in a cytochrome-oxidase system (*left*) and of hydrogen peroxide utilization in a peroxidase system (*right*). O_2 utilization is measured by means of a platinum microelectrode polarized as a cathode and the hydrogen peroxide utilization is measured with the electrode polarized as an anode. The cytochrome-oxidase system used was that obtained from Keilin and Hartree heart muscle preparation. The peroxidase was crystalline material (*exper. 574-DE*).

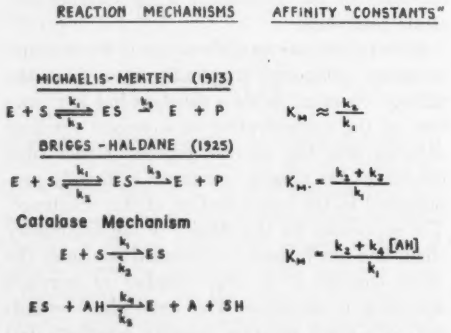
REACTION MECHANISMS
AFFINITY "CONSTANTS"
MICHAELIS-MENTEN (1913)
$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$
$K_M \approx \frac{k_3}{k_1}$
BRIGGS-HALDANE (1925)
$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$
$K_M = \frac{k_3 + k_2}{k_1}$
Catalase Mechanism
$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES$
$K_M = \frac{k_2 + k_4 [AH]}{k_1}$
$ES + AH \underset{k_3}{\overset{k_4}{\rightleftharpoons}} E + A + SH$

FIG. 2. Stages in the development of relationships for enzyme-substrate affinity of a one-enzyme one-intermediate system. Arrow length signifies magnitude of velocity constants on logarithmic scale.

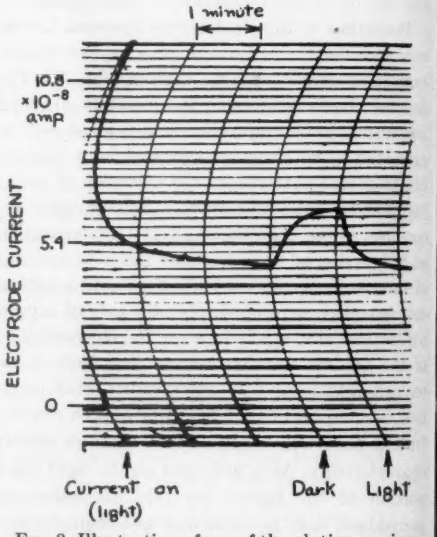

FIG. 3. Illustration of use of the platinum microelectrode in measuring reversal of carbon monoxide inhibition of respiration caused by illumination. The electrode, polarized at a cathode, is inserted into the suspension of yeast cells in an atmosphere of 20% O_2 and 80% CO. From *J. Biol. Chem.* (073).

in an atmosphere of oxygen and carbon monoxide (2). After a few minutes a steady state of oxygen concentration is established in the illuminated drop. Cessation of illumination causes a diminution of respiration and a rise of oxygen concentration in the drop. This effect is reversible. The effectiveness of various wavelengths of light in relieving the CO inhibition of respiration is different and Warburg first obtained the absorption spectrum of the CO compound of the terminal oxidase in this manner (3). The platinum microelectrode method is much more sensitive than the manometric one and reveals a complete spectrum for the carbon monoxide compound of the terminal oxidase of a suspension of mouse ascites tumor cells in the wavelength range 400–650 mμ (2). The spectrum of figure 4 shows a sharp absorption band at 430 mμ, with subsidiary bands at 550 mμ and 590 mμ.

While Keilin described various types of cytochromes as early as 1925 (4), it was many years

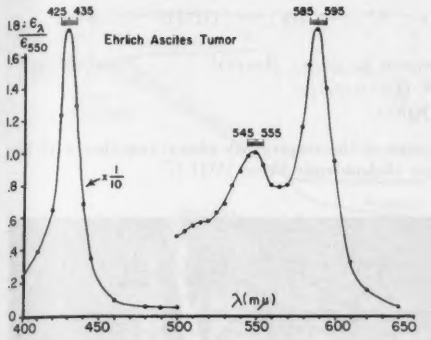

FIG. 4. Relative efficiency of various wavelengths of light in relieving the CO inhibition of respiration of a suspension of Ehrlich ascites tumor cells. The platinum microelectrode method illustrated by fig. 3 is used. From *J. Biol. Chem.* (074).

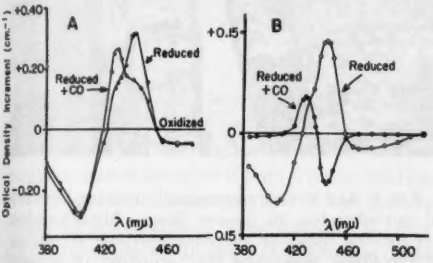

FIG. 5. Spectra representing the difference between reduced and oxidized forms of cytochrome components. *Solid circles* represent the difference between reduced and oxidized forms of cytochrome; *open circles* give the difference between the reduced forms which have been treated with carbon monoxide and the oxidized forms. In *part B*, the curve following the solid circles represents the difference between the two spectra in *part A*. Keilin and Hartree heart muscle preparation. From *J. Biol. Chem.* (*exper. 958-3*).

before he was able to identify spectroscopically such a carbon monoxide compound in his cytochrome system. In 1939 Keilin and Hartree (5) observed spectroscopic shifts on adding carbon monoxide to their heart muscle preparations which they attributed to the cytochrome a_3-CO compound. These spectra are now clearly represented in more recent studies (fig. 5 and refs. 6-8). In *part A*, the solid circles represent the absorption bands of the reduced cytochromes, that of a_3 being at 445 mμ. Addition of CO causes a new band to appear at 430 mμ and one to disappear at 445 mμ. The difference between the two spectra in *A* is given by the solid circles in *B*. Since 430 mμ is the peak of the photochemical

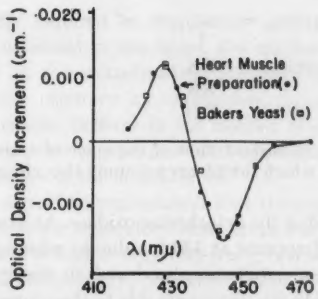

FIG. 6. Photodissociation spectra (difference spectra). Spectroscopic changes caused by cessation of illumination of carbon monoxide compound of cytochrome a_3 of heart muscle and yeast. Absorbancy increases and decreases are those recorded spectrophotometrically upon cessation of illumination of the carbon monoxide compound with red light (*expers. 931* and *934*).

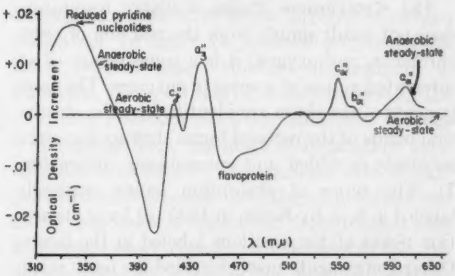

FIG. 7. Absorption bands of respiratory enzymes of excised frog satorius muscle. The changes of light absorption that occur when aerobic frog sartorius muscle becomes anaerobic are illustrated by this graph. Absorption peaks are designated by a letter, in accordance with the notations of Keilin (4). The absorption maxima are at 605 for cytochrome *a*, 562 for cytochrome *b*, 551 for cytochrome *c* and c_1, 445 for cytochrome a_3. Also shown are the absorption peaks due to pyridine nucleotide reduction at about 340 mμ, and the distinctive trough in the region of 460 mμ due to the reduction of oxidized flavoprotein. This spectrum is from unpublished data of Dr. A. M. Weber obtained in this laboratory (W1).

action spectrum, it can be justifiably attributed to the CO compound of the terminal oxidase. Since the 445 mμ band is diminished in this reaction, it would then represent the reduced form of the terminal oxidase. Keilin and Hartree were, however, unable to photodissociate the 430-mμ CO compound, but we have recently found appropriate conditions for accomplishing this, and a typical result is shown in figure 6 (9). This figure shows the absorbancy changes that occur upon cessation of illumination of the carbon monoxide

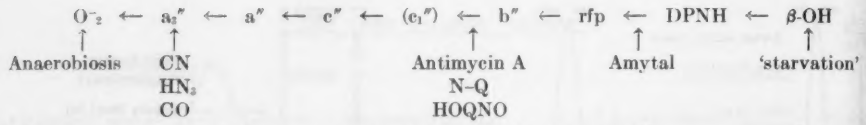

FIG. 8. A current view of sequence of action of enzymes of the respiratory chain, together with the points at which inhibitors act upon this chain. Courtesy of Academic Press (MD-47).

compound of the cytochrome oxidase. An absorption band appears at 430 mμ due to reformation of the dissociated compound and an absorption band at 445 mμ disappears due to the conversion of the reduced form of the oxidase into its carbon monoxide compound. Thus it is now conclusively proved that the cytochrome having an absorption band at 445 mμ is the one responsible for the formation of a photodissociable carbon monoxide compound to which we can attribute the inhibition of cellular respiration.

The Cytochrome Chain. Cellular respiration does not result simply from the reaction of cytochrome a_3 and oxygen; it is a consequence of an integrated action of a series of enzymes. The components of the chain are identified by the absorption bands of the reduced forms that appear when substrate is added and anaerobiosis ensues (fig. 7). The series of absorption bands originally labeled a, b, c, by Keilin in 1925 (5) have absorption peaks at the positions labeled in the figure. Other components now identified by more sensitive spectrophotometric methods are included in this spectrum. This pattern of absorption bands is characteristic of most cells; other patterns are found in microorganisms. Studies by Keilin and Slater (10), Ball (11), Slater (12) and in this laboratory led to the identification and to the sequence of action of seven components of the respiratory chain (fig. 8). The sequence of action of the cytochromes is best determined by measuring the relative speeds with which the absorption bands of the reduced compounds disappear upon rapidly adding oxygen (13), that of the terminal oxidase first, the second member, second, etc. In addition to this method, it is possible to use experimental conditions or selective inhibitors that interrupt the action of the respiratory chain (fig. 8) at each end, by starvation or by anaerobiosis, at the site of the oxidase by cyanide, carbon monoxide, or azide, or, more usefully, at points within the chain between b and c (12) or between fp and DPNH (14). Inhibitors for only two of the six places between the components are known, yet these inhibitor data agree with data based on kinetic studies and provide an acceptable hypothesis for the current sequence of action of the respiratory enzymes.

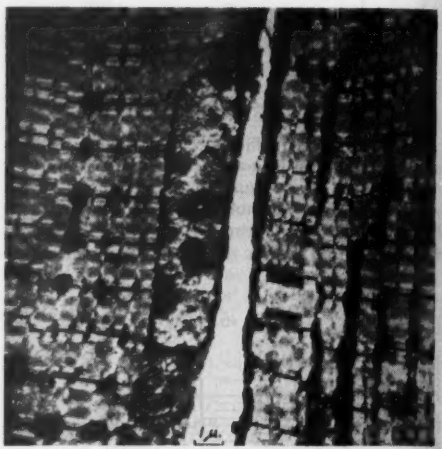

FIG. 9. An electron micrograph showing location of mitochondria in muscle fibers. Mitochondria are located opposite the characteristic striations of the fibers. Sjöstrand (15), courtesy of *Science Tools*.

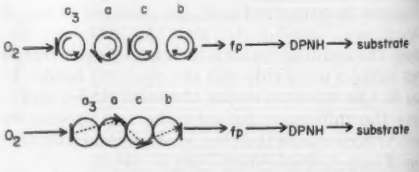

FIG. 10. A schematic representation of two ways for interaction of adjacent members of the respiratory chain in order to permit transfer of electrons. Courtesy of Interscience Publishers (MD40-41).

Structure of Respiratory Chain. The chain of enzymes responsible for cellular respiration is embedded in the structure of the cell particle or the mitochondrion. An electron micrograph obtained by Sjöstrand (15) illustrates the location of mitochondria with respect to the characteristic bands of the muscle fibers (fig. 9).

The cytochromes are attached to the internal structure of the mitochondria and may be a part

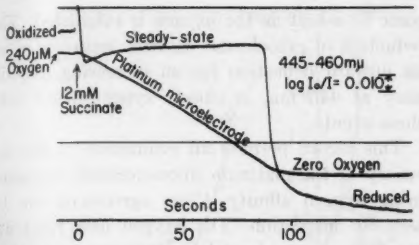

FIG. 11. Comparison of O_2 utilization and cytochrome kinetics in a Keilin and Hartree succinic-oxidase preparation. The platinum electrode polarized at the cathode is used to record kinetics of initiation of respiration upon addition of succinate and its termination upon the exhaustion of the dissolved O_2. The spectrophotometer, adjusted to measure the absorbancy differences between 445 and 460 mμ, records the oxidized state of cytochrome a_3 prior to addition of succinate, the steady state in the presence of succinate and oxygen, and the complete reduction of the oxidase when the O_2 dissolved in the solution is exhausted (*expt. 222-B*).

of their double-walled internal morphology called cristae (16). Under such conditions free diffusion and collision of the enzymes with one another cannot occur as freely as if they were in solution. A very schematic representation of two possible means of interaction of these enzymes is represented by figure 10. In these diagrams the circles represent the enzyme proteins. In one mechanism, thermal vibrations of the enzymes or rotations about their point of attachment to the cell structure are assumed to be the mechanism for interaction of these enzymes. Calculations indicate that roughly 10^7 of such collisions would occur due to thermal energy each second, a number more than adequate to account for the electron transport rate of roughly 10^2; 30 per second are usually required by respiratory activity. As is also indicated in the figure, the enzyme may be fixed in the mitochondrial structure and may transfer electrons by conduction through peptide chains, perhaps in the manner of semi-conductors, but in a manner that all electrons pass the hemes of the cytochromes.

It should be remembered that the over-all activity of the respiratory chain is used in the production of high-energy phosphate bonds and the process of oxidative phosphorylation occurs at 3 or more sites in the respiratory chain. Recent spectroscopic studies indicate that the oxidation-reduction couples DPNH-fp, b-c, and c-a are those involved in the energy conservation process (17) of oxidative phosphorylation.

Spectroscopic Studies of the Cytochrome Chain in Operation. Sensitive spectrophotometers have been developed for the purpose of recording the action of cytochromes in the process of oxygen utilization. In figure 11 particles prepared from heart muscle cause oxidation of succinate to fumarate. The recording of oxygen tension by the vibrating platinum electrode indicates, without a stirring artifact, the start of respiration upon addition of succinate. The constant rate of oxygen utilization proceeds until the oxygen dissolved in the suspension is exhausted. Synchronized with these respiratory changes are changes in the oxidation-reduction levels of the terminal oxidase. Upon addition of succinate, a downward deflection of the trace occurs. This corresponds to an increased absorbancy at 445 mμ and means that a partial reduction of the terminal oxidase has occurred. The extent of reduction remains roughly constant in the steady state interval of respiration. Upon termination of respiration, an abrupt and rapid reduction of the terminal oxi-

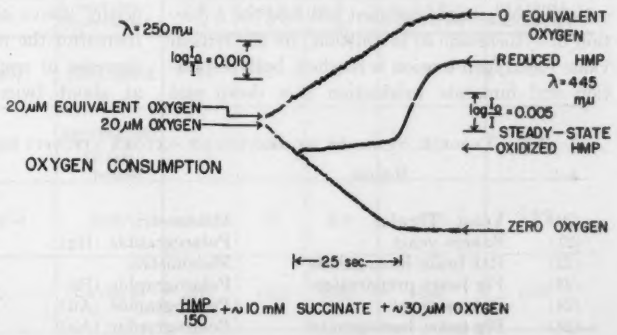

FIG. 12. Simultaneous recordings of O_2 utilization by the platinum electrode and fumarate production measured spectrophotometrically. Reduction of cytochrome a_3 is also measured spectrophotometrically in a Keilin and Hartree heart muscle preparation. This record was obtained by a repetition of the experiment under identical conditions with the ultraviolet recording of the fumarate production or with a recording of the reduction of cytochrome a_3 at 445 mμ. In this record the reduction of cytochrome a_3 is indicated as an upward deflection of the trace (*exper. 597-1011*).

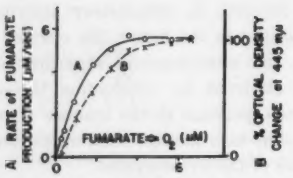

FIG. 13. A replot of the data of fig. 12. *Trace A:* rate of fumarate production versus change of fumarate concentration or its equivalent in O_2 concentration (O_2 = 2 fumarate). In *trace B* the extent of reduction of cytochrome a_3 is plotted as a function of O_2 concentration (*exper. 597-11, 13*).

dase occurs and it soon becomes completely reduced. The recordings illustrate 'critical oxygen tension' and 'half-maximal oxygen tension.' Above the critical oxygen tension, the oxidase is in a steady state; the amount of its reduction in this state depends upon the supply of substrate and not upon the supply of oxygen. As soon as the oxygen concentration falls below the critical level, the oxidation of the oxidase becomes dependent upon the oxygen concentration and under these conditions it is rapidly reduced.

In particles prepared from heart muscle which oxidize succinate to fumarate, the oxidase properties can be measured not only in terms of oxygen disappearance as recorded by the platinum microelectrode, but also by fumarate production since fumarate has an ultra-violet absorption band at 250 mμ. Thus in figure 12 we show a combination of the platinum electrode record showing oxygen disappearance and a spectrophotometric record showing fumarate production. In addition, the absorption of the terminal oxidase is measured at 445 mμ. The reaction, having already been started by the addition of succinate in the presence of oxygen, is in a steady state when the record begins. Thus the oxygen and fumarate reactions proceed at a constant rate and the reduction of cytochrome a_3 is constant. As the critical value for oxygen tension is reached, both respiration and fumarate production slow down and come to a halt as the oxygen is exhausted. The reduction of cytochrome a_3, here represented as an upward deflection for an increasing absorbancy at 445 mμ, is closely synchronized with these events.

This record permits an evaluation of the accuracy of the platinum microelectrode in measuring oxygen affinity. Close agreement can be seen by inspection. The oxygen and fumarate traces are approximately mirror images as they should be, although the fumarate trace is the more accurate of the two.

In order to determine the oxygen requirements for this system, it is useful to plot the slope of these traces against fumarate concentration or its equivalent in oxygen concentration. Figure 13 gives in *curve A* the decrease of fumarate rate as the oxygen concentration falls to zero. Above 4 μM oxygen the rate is constant and this defines the critical oxygen concentration. Below 4 μM an abrupt decrease of rate occurs and the half-maximal value is reached at about 1 μM oxygen, in reasonable agreement with the values of 2 μM oxygen given earlier (23) and of 0.5 μM oxygen obtained recently by Longmuir (18) and by Baender and Kiesse (19) for more slowly respiring systems, and earlier by Winzler for yeast cells. (For a summary, see table 1.)

It should be noted that the very low values obtained by Longmuir in 1954 (24), which were in disagreement with our earlier values (23), are not confirmed by these later studies. The 100-fold smaller values of K_m that Longmuir obtained were probably due to inactivation of the preparation either prior to or during the actual experiment.

Curve B of figure 13 represents the increasing reduction of the oxidase as the oxygen concentration falls to zero. An appreciable reduction occurs above the critical oxygen tension, and thereafter the reduction of the oxidase leads the decrease of respiration rate and is half-reduced at about twice the oxygen concentration re-

TABLE 1. SUMMARY OF RESULTS ON OXYGEN AFFINITY OF RESPIRATORY CHAIN

Ref.	Material	Method	Temp., °C	'Km' mm Hg	'Km' μM
(20)	Yeast (Torula)	Manometric	10	~9	16
(21)	Bakers yeast	Polarographic (Hg)	20	0.5	0.9
(22)	Rat brain homogenate	Manometric	37	<4	<7
(23)	Pig heart preparation	Polarographic (Pt)	25	1	2
(24)	Bakers yeast	Polarographic (Au)	~18	0.3	0.6
(24)	Pig heart homogenate	Polarographic (Au)	~18	0.01	0.02
(19)	Rat liver mitochondria	Manometric	20	0.5	0.8
(18)	Rat liver cells	Polarographic (Au)	40		0.5

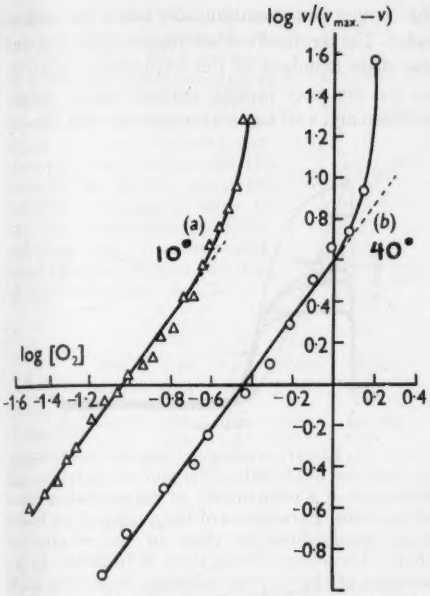

FIG. 14. Longmuir's data on the relationship between respiratory activity and O_2 concentration for liver cells plotted in a logarithmic scale (18). *From Biochem. J.*

quired for half-maximal respiratory activity. Thus the oxidase is more sensitive to oxygen than is the respiratory rate. This apparent inconsistency is explained in terms of the mechanism proposed below.

Baender and Kiesse (19) and Longmuir (18) have plotted the logarithm of the respiratory activity against the logarithm of oxygen concentration and find that the function varies as the 1.4 power of the oxygen concentration, as is illustrated by Longmuir's figure (fig. 14). Participation of oxygen to the first power of this reaction would have resulted in 45° straight line. This result suggests an anomaly in the reaction of oxygen with the oxidase, and an explanation for this is afforded by the mechanism proposed below.

Effect of Metabolic State on Oxygen Affinity of Respiratory Chain. Few investigations of oxygen affinity have taken into account the various metabolic states of the cell. Physiologists recognize a number of states of metabolism, particularly those associated with rest and activity, as well as those established by hormonal control. Four states can be assigned biochemical definitions in isolated mitochondria (see table 2). Associated with these states are characteristic oxidation-reduction levels of the cytochrome components. In the starved state, characterized by an excess of ADP and a lack of substrate, all of the cytochromes are oxidized and the respiration rate approaches zero. In the active state, characterized by the presence of ADP and substrate, the respiratory rate is fast and the limitation to respiration is set by the properties of the respiratory chain. The steady-state reduction of those components near oxygen is slight and increasing values are observed for those nearer the substrate end of the respiratory chain. In the resting state, characterized by the presence of substrate and the lack of ADP or inorganic phos-

TABLE 2. STEADY STATE OF RESPIRATORY ENZYMES

CHARACTERISTICS					STEADY STATE % REDUCTION				
					COMPONENTS				
STATE	ADP LEVEL	SUBSTRATE LEVEL	RESPIRATION RATE	RATE LIMITING COMPONENT	CYTOCHROME a	CYTOCHROME c	CYTOCHROME b	FLAVO-PROTEIN	PYRIDINE NUCLEOTIDE
STARVED	+	−	SLOW	SUBSTRATE	0	0	0	0	0
ACTIVE	+	+	FAST	RESPIRATORY CHAIN	<4	6	16	20	53
RESTING	−	+	SLOW	ADP	~0	14	35	40	>99
ANAEROBIC	+	+	0	OXYGEN	100	100	100	100	100

Rat liver mitochondria. Substrate—hydroxybutyrate. Phosphate acceptor—ADP, 25°.

TABLE 3. EFFECT OF TRANSITION FROM RESTING TO ACTIVE STATE ON OXYGEN AFFINITY OF KIDNEY MITOCHONDRIA (EXPER. 712)

State of Mitochondria	Q_{O_2} $\mu l/mg/hr$	K_m μM
3 (Active)	130	1.6
4 (Inactive)	26	1.1

Succinate as substrate. Temp., 25° C.

phate, the components nearer oxygen become somewhat more oxidized while those nearer substrate become more reduced, especially pyridine nucleotide, the reduction of which exceeds 99%. Lastly, the anaerobic state gives, by definition, complete reduction of the components of the respiratory chain.

It is of considerable interest to know whether or not the critical oxygen tension and oxygen affinity are the same or different in active and resting states. Elementary considerations would lead us to conclude that a considerable increase of critical oxygen tension would occur in activity. But a study of the reaction mechanism for oxidative phosphorylation (17) shows that more of the respiratory enzymes are available in the active state than in the quiescent state. Thus not much change of oxygen affinity would be expected. This is verified by table 3 in which a change of Q_{O_2} from 26 to 130 for kidney mitochondria causes only a 50% increase of the oxygen concentration for half-maximal respiratory rates. Based upon this result, one might predict that hormones or related substances which exert their effect by an uncoupling of phosphorylation would have a relatively small effect upon the oxygen affinity.

Reaction Mechanisms Underlying Critical Oxygen Tension and Oxygen Affinity. The chemical processes representing the reaction of the terminal oxidase with oxygen and the members of the chain with each other can be written in the form of nonlinear differential equations (17). Such equations can be solved by means of an electric analogue computer and solutions for the response of this enzyme system to changes of oxygen concentration have been obtained (fig. 15). The curve labeled x corresponds to the oxygen concentration which is suddenly raised to an initial level of 40 μM (see scale on right side of figure) and is then expended in respiration in about 4 seconds. This curve is similar to the platinum electrode data obtained experimentally and consists of a linear portion in which the oxygen is utilized at a constant rate. This is followed by an abrupt decrease in respiratory rate when the oxygen concentration falls below the critical value. The terminal oxidase represented by p_1 and the other members of the respiratory chain, p_2, p_3, p_4, are very rapidly oxidized upon oxygen addition and a steady state ensues during the me-

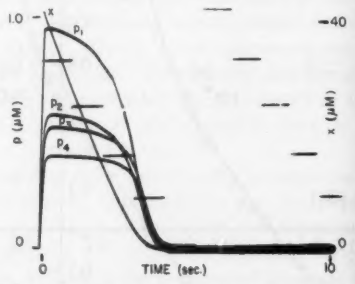

FIG. 15. Electric analogue computer solution for the kinetics of O_2 utilization and of oxidation and reduction of 4 components of an oxidative chain of enzymes. Parameters of the analogue are based upon observations of those of the respiratory chain. The computer solution is initiated by an increase of the O_2 concentration from zero to 40 μM (see scale on right-hand side of figure). Concentration of each one of the enzymes represented is 1 μM and the extent of oxidation of these 4 components upon the addition of 40 μM O_2 is indicated by scale on left-hand side of the curve. Higgins, Holmes and Barr, unpublished data (AC-54).

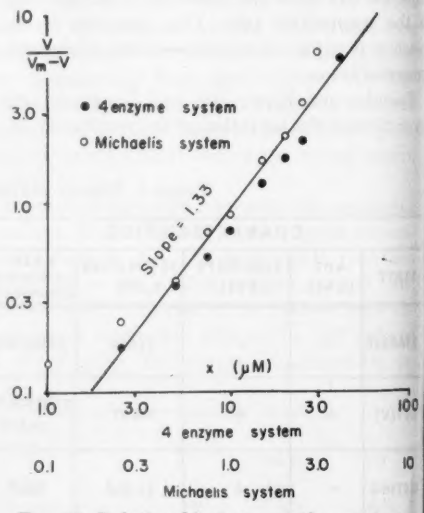

FIG. 16. Relationship between the respiratory activity and the O_2 concentration as derived from the analogue computer data. This curve and that of fig. 14 are in logarithmic coordinates. The slope of the curve corresponds to an exponent of 1.33 in the O_2 concentration. Higgins, Holmes, and Barr, unpublished data (AC-71).

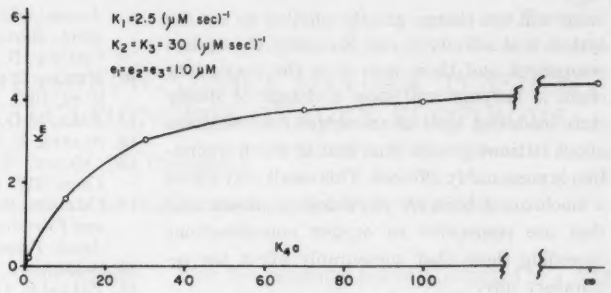

FIG. 17. Effect of substrate concentration (a) upon O_2 requirement (K_m) for a sequence of 3 enzymes. As indicated in the figure, the concentration of 3 enzymes is identical and equal to 1 μM. Velocity constants for reaction between the components (K_2, K_3) and of the terminal component with O_2 are also indicated (K_1). Unpublished data from the studies of Higgins, Holmes and Barr of this laboratory (AC-73).

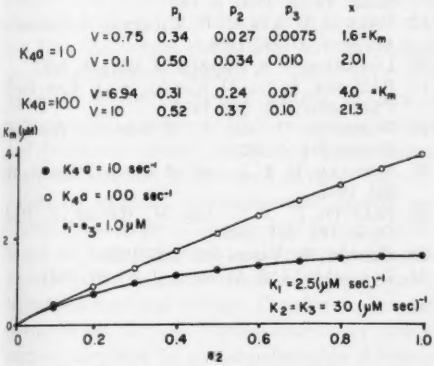

FIG. 18. Effect upon the O_2 requirement (K_m) with a variation of the middle enzyme in a chain of 3 enzymes for 2 values of the substrate concentration K_{4a} = 10 *solid circles*, K_{4a} = 100 *broken circles*), other conditions similar to those of fig. 17. Unpublished data from the studies of Higgins, Holmes and Barr of this laboratory (AC-72).

tabolism of the oxygen. The oxidase (p_1) starts to become more reduced (downward deflection of the p_1 trace) at oxygen concentrations above those corresponding to any perceptible diminution of the rate of oxygen utilization and then becomes rapidly reduced as the oxygen is exhausted. On the other hand, the fourth enzyme of this sequence, p_4, stays in a relatively constant steady state until the critical oxygen tension is reached and then its concentration falls in close synchronism with the decrease of respiratory rate. Changes in oxidation-reduction level of the terminal oxidase and of members of the chain near it occur at oxygen concentrations considerably exceeding the critical level based upon respiratory activity. An explanation of this increased reduction of the terminal oxidase without a corresponding change of respiration is that there is an overabundance of the terminal oxidase in the mammalian respiratory chain. Thus its oxidation level may change considerably and leave nevertheless an adequate amount of the reduced form of the oxidase to carry on the respiratory process without a measurable change in the respiratory rate. It is a protective or cushioning effect of the cytochrome chain—a unique property which has not received the attention that it deserves. This phenomenon may be of considerable significance physiologically and may afford an explanation for the failure of certain physiological functions at oxygen tensions exceeding the critical value for respiration. Such processes might be triggered by the response of the terminal oxidase to oxygen concentrations and not by the net rate of oxygen utilization. A mechanism for such triggering is unknown.

An explanation for the anomalous 1.4 power relationship between oxygen concentration and the respiratory activity found experimentally is afforded by the computer data. In figure 16 we have plotted the logarithm of respiratory activity versus the logarithm of the oxygen concentration for the analogue computer data and we find that the computer solutions give an exponent of 1.33, in reasonable agreement with the experimental exponent of 1.4. In this mechanism we have assumed no peculiar equilibrium of oxygen and the terminal oxidase, only an essentially irreversible combination of oxygen with the oxidase. Thus it is in the nature of the cytochrome system to give a sharp break in the respiratory activity-oxygen concentration relationship without the postulation of any unusual oxygen-oxidase stoichiometry.

SUMMARY

Two general ideas presented here may be of some use to the physiologist. First, the oxygen affinity of the respiratory system is so high that no changes of rate will occur until the oxygen concentration has fallen to about 4 μM at 25°. This

value will not change greatly whether or not the system is at activity or rest. Secondly, the oxidase component and those near it in the respiratory chain of enzymes will show a change of steady state oxidation level at an oxygen concentration about 10 times greater than that at which respiration is measurably affected. This result may afford a biochemical basis for physiological phenomena that are responsive to oxygen concentrations exceeding those that measurably affect the respiratory rate.

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METHODS FOR MEASURING TISSUE OXYGEN TENSION; THEORY AND EVALUATION: THE OXYGEN ELECTRODE

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THE oxygen electrode is a polarographic device for measuring the concentration of oxygen dissolved in the medium in which the tip of the electrode is placed. Perhaps it would be better to say that the oxygen electrode is but one part of an electrochemical system in which oxygen is caused to be electrolytically reduced, the rate of reduction being measured by the electrode current and being determined by, among other things, the concentration of dissolved oxygen. The elements of such an electrochemical system are illustrated in figure 1. Immersed in tissue or solution is the bare tip of a platinum wire which is connected to the negative terminal of a suitably adjustable source of voltage. The electrical circuit is completed through a galvanometer or direct-current amplifier to a non-polarizable reference electrode such as a calomel half-cell or a silver-silver chloride electrode. When the platinum is several tenths of a volt negative to the calomel half-cell, oxygen dissolved in the medium is electrolytically reduced at the surface of the platinum. Figure 2 shows the magnitude of the reduction current as a function of the polarizing potential (1). The upper curve shows that in a solution saturated with air the current, or the rate of reduction, increases as the polarization voltage is increased. The disappearance of oxygen at the electrode surface permits oxygen to diffuse towards the surface and establishes a concentration gradient or diffusion field adjacent to the surface. At potentials more negative than about 0.6 v. electrolysis is sufficiently rapid that all of the oxygen diffusing to the surface is reduced, and the rate of the reaction becomes limited by diffusion. This limitation results in the plateau in the curve in the region of 0.6 to 0.8 v. At still greater negative potentials the current increases again as hydrogen ions are discharged to form molecular hydrogen. In the absence of dissolved oxygen, the current-voltage relationship is shown by the lower curve in which the electrode current is zero until hydrogen begins to be discharged. With the polarizing potential adjusted to a value in the plateau region, about 0.7 v., the electrode may be calibrated by measuring the current to the electrode when the surrounding medium is equilibrated with different gas mixtures of known oxygen content. Such a calibration curve is illustrated in figure 3, for a case in which an electrode was located in a capillary tube through which solution flowed at constant velocity (2). This shows that the magnitude of the current in the plateau region is directly proportional to the concentration of dissolved oxygen or to the oxygen tension of the equilibrating gas.

This brief, rather oversimplified statement of the operation of the oxygen electrode is not meant to be misleading. There are limitations that must be considered carefully in specific applications. Two points may be emphasized now, however. One is that measurements with an oxygen electrode are largely empirical; the electrode must be calibrated under conditions essentially the same as those used experimentally. The second point is that the oxygen electrode does not operate as a reversible potentiometric device for measuring the activity of a chemical component, as does the hydrogen electrode or the chloride electrode, but it acts rather as a so-called amperometric device which irreversibly consumes the material whose concentration it is measuring. It is for this reason that some authors prefer to call the device an oxygen cathode rather than an oxygen electrode.

It is not possible to discuss here the many biological applications of this versatile device and the limitations that must be considered in relation to each. Use has been made of the oxygen electrode in cell suspensions, in solutions of reacting enzymes, in blood and in plasma, *in situ* directly in contact with the surfaces of tissues or implanted in tissues, and in a variety of respirometers. Considering the electrode in each application as an instrument, one can ask for statements of the properties or specifications of the instrument. Such statements usually describe sensi-

tivity, response time, linearity and stability, and in the case of the oxygen electrode they should also answer questions concerning possible secondary effects of the consumption of oxygen and the production of hydroxyl ions at the electrode surface, concerning sensitivity to pH, to temperature and to movement and concerning possible reduction of constituents other than oxygen. In addition, any complete discussion of the interpretation of electrode-current records, particularly of those made with the electrode in contact with metabolizing tissues, would require the presentation of the elements of diffusion theory.

Some basis for understanding possible limitations in the use of the oxygen electrode may be provided by a further consideration of the events that take place at or near the surface of the electrode. It has been pointed out that in the plateau region of the current-voltage curve, the reaction at the electrode is limited by the rate at which oxygen diffuses to the surface from the surroundings. The curve in figure 4 describes the steady-state diffusion field in the neighbourhood of a spherical electrode of radius R_O, in an infinite, homogeneous medium originally containing oxygen at a concentration C_O (3). With an electrode of different shape, located in a finite system, the diffusion field will be quantitatively different from this, ending at the boundaries or at the sources of supply of oxygen. The electrode current is proportional to the integral over the electrode surface of the normal component of the oxygen gradient there. Anything which modifies this diffusion field changes the electrode current. Such modification might result from *1*) slight movement of the electrode with respect to the medium, thus replacing the oxygen deficient layer at the surface with a layer containing more oxygen; *2*) a local change in the solubility or in the diffusion coefficient of oxygen; *3*) the entrance of an oxygen-bearing red blood cell into the field; *4*) a change in oxygen tension at the boundaries of the system; and/or *5*) a change in the metabolic rate of the system.

The time required for an electrode to respond to a change in oxygen concentration in the surrounding medium is difficult to state, or even to define theoretically. Insofar as the diffusion field is largely confined to the immediate vicinity of the electrode surface, the readjustment of the diffusion field is relatively fast, involving small distances, but to the extent that the field has a

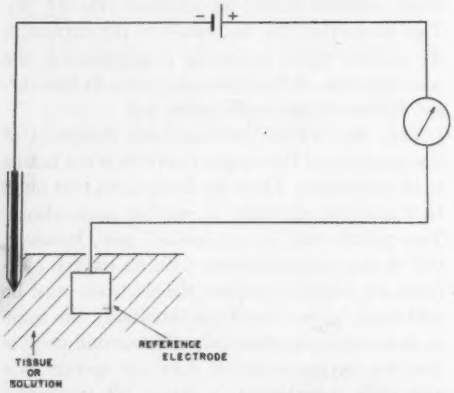
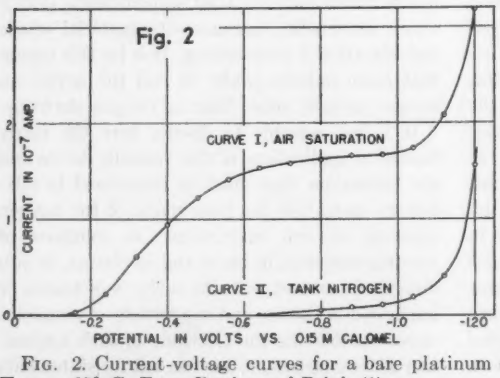


FIG. 1. Elements of electrolysis circuit incorporating an oxygen electrode.



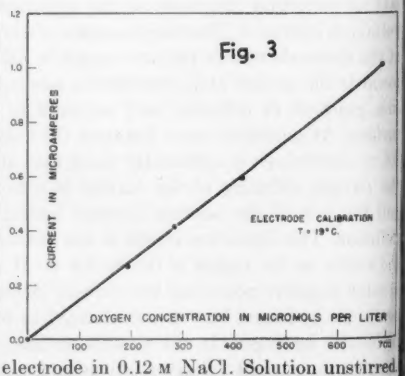


FIG. 2. Current-voltage curves for a bare platinum electrode in 0.12 M NaCl. Solution unstirred. Temp., 25° C. From Davies and Brink (1).

FIG. 3. Calibration curve for a bare platinum electrode in Ringer's solution. Replotted from Carlson, Brink and Bronk (2).

fringe that extends to the boundaries of the system, the approach of the current to a final steady value may be slow. A bare electrode begins to respond almost instantaneously and is well suited for detecting and measuring approximately, very rapid changes in oxygen concentration. Various types of coated electrodes respond with a lag which is related to the equilibration time-constant for oxygen in the coating layer. Because a large proportion of the diffusion field is confined to the coating layer on such electrodes, they are much less prone to movement artifact than are bare electrodes. Chance (4) and others studying solutions or cell suspensions have minimized limiting effects of the diffusion field, yet retained rapid response, by rapidly vibrating or rotating a bare electrode, a procedure which also increases the sensitivity (measured in amperes per unit oxygen concentration).

Perhaps the greatest defect of the oxygen electrode—certainly a property that has dissuaded faint-hearted investigators from its use—is the tendency of the electrode current to decrease gradually over a period of tens of minutes or hours, even though the oxygen tension in the solution or tissue is maintained at a standard level. This decline in sensitivity is called 'aging.' A bare electrode may age at a rate of several percent per hour. Following are listed two reactions, the first or both of which are thought to take place at the metal surface (1, 3):

$$O_2 + 2H^+ + 2e \rightarrow H_2O_2 \qquad (1)$$

$$H_2O_2 + 2H^+ + 2e \rightarrow 2\,H_2O \qquad (2)$$

If only the first occurs, two electrons flow in the circuit for each oxygen molecule that reacts. Davies and Brink (1) observed a 2-electron reaction in one of their electrodes. On the other hand, they found a 4-electron reaction in other electrodes, and D. K. Hill (5) by a different method, also observed a 4-electron reaction. The latter observations indicate that any peroxide formed in the first reaction was immediately reduced to water by the second. Lack of evidence for fractional electron reactions in the range of 2-4 electrons per oxygen molecule makes it seem unlikely that any particular electrode shifts gradually from a 4-electron to a 2-electron reaction, with a resulting temporal decrease in sensitivity.

One obvious, possible cause of aging, however, is the occlusion of part of the electrode surface by precipitation upon it of insoluble salts. Both

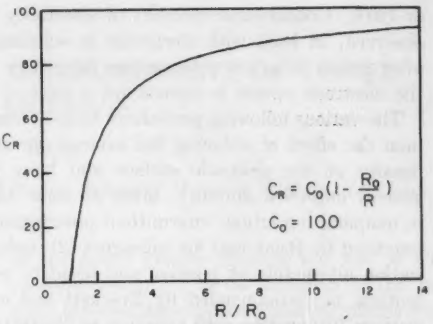


FIG. 4. Curve describing concentration of oxygen, C_R, as a function of distance, R, from the center of a spherical oxygen electrode of radius R_0. Steady state conditions after infinite time, in infinite homogeneous medium initially containing oxygen at concentration C_0. Relationship derived by Kolthoff and Lingane (3).

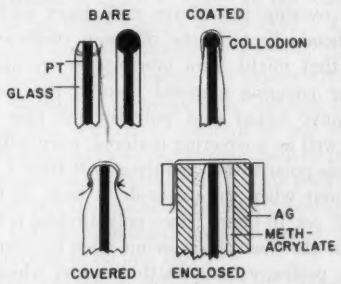


FIG. 5. Several types of oxygen electrode, in cross-section.

reactions 1 and *2* require the medium near the electrode to supply hydrogen ions. In the physiological pH range it would have been more meaningful if the reactions had been written as producing hydroxyl ions. The reality of base production is easily demonstrated by the addition of an indicator, e.g. phenol red, to the solution flowing past a functioning electrode. With some degree of alkalinity prevailing close to the electrode surface there may be precipitated insoluble salts of calcium, magnesium, carbonate, phosphate, and perhaps proteinate, either from physiological solutions or from tissue fluids. Spongy or flaky deposits have occasionally been seen on the tips of electrodes after use. Since the rate of hydroxide production and therefore the intensity and extent of alkalinity varies with the electrode current, it is not surprising that an electrode may operate quite stably in media equilibrated with 20 or 30% oxygen but may begin to age rapidly if the oxygen mixture is increased to 50

or 100%. Considerable recovery of sensitivity is observed, at least with electrodes in solutions, after return to a lower percentage oxygen or if the electrode circuit is opened for a time.

The various following procedures have in common the effect of reducing the average current density at the electrode surface and have in general improved stability, often at some loss in temporal resolution: intermittent polarization, practiced by Brink and his colleagues (2); polarization alternately at positive and negative potentials, as recommended by Brackett and coworkers (6); coating with collodion as illustrated in figure 5, at the upper right; and covering with a film of cellophane as described by Clark and colleagues (7) and illustrated at the lower left in figure 5. (See also ref. 8 for a discussion of substances in blood affecting the calibration of a cellophane-covered electrode.) The coating and covering procedures may have additional beneficial effects if the diffusion coefficients of ions that might form precipitates are also less in the covering material than in the medium. We have found that polyethylene film serves very well as a covering material, even with continuous polarization, reducing drift from 1 or 2% per hour when the electrode is bare, to 0 to 2 or 3% per 10 hours. Since polyethylene is hydrophobic the electrolysis circuit must be completed via a pathway underneath the film where it is tied. This also means that precipitating ions will slowly diffuse to the electrode surface via this pathway.

Last year at Atlantic City, Clark (9) described an oxygen electrode which was enclosed, together with its reference electrode, behind a polyethylene film. Reeves, in another paper in this symposium, describes the performance of such an electrode. One possible version is illustrated at the lower right in figure 5. The enclosed electrode has many possible uses. It will measure the oxygen tension in a gaseous phase or in solutions containing high concentrations of ions that would precipitate at the surfaces of previously described electrodes. The procedure of isolating the electrochemical measuring circuit from the surrounding fluids or tissue appears to have almost solved the problem of drift in electrode sensitivity.

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GASOMETRIC METHOD FOR MEASUREMENT OF TISSUE OXYGEN TENSION

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THIS method is probably the oldest approach for estimating the oxygen and carbon dioxide tension of tissues. It consists of introducing a bubble of gas into the tissues desired. This gas pocket is then allowed to equilibrate and subsequently removed for gas analysis. The technique is simple, direct and can be applied to unanesthetized animals and man. While it is thus relatively easy to obtain a value for O_2 tension of the gas in the pocket, it does not necessarily follow that this is the O_2 tension of the tissue. The difference between these two values furnishes the basis for our discussion.

An *in vitro* method can also be used for fluid tissues and secretions. The fluids are removed anaerobically, equilibrated *in vitro* with a small gas bubble which is subsequently analyzed for its gas content. This method is usually applied to the analysis of blood gas tensions and will not be further discussed.

Total Gas Pressure in Tissue Gas Pockets. In order to interpret the value of the O_2 tension found in a tissue gas pocket, one is forced to consider the behavior and physical properties of all the gases present. This usually includes CO_2 and N_2. Measurements of the total gas pressure of a tissue gas pocket indicate that they are very close to atmospheric pressure. As these gases are slowly resorbed, the pressures do not change. Even pressures of 2–5 cm H_2O, whether above or below atmospheric pressure, are negligible from the point of view of dynamic gas exchange. For practical purposes, therefore, we can assume that total gas pressure in tissue pockets remains essentially atmospheric.

Total Gas Pressure in Tissues. Each of the gases found in the tissues exerts a partial pressure and the sum of these partial pressures can be called the total gas pressure of the tissues. This total gas pressure must always be less than atmospheric pressure when tissues are metabolizing. This is brought about firstly by difference in the slopes of the O_2 and CO_2 dissociation curves. For a normal gas exchange, the fall in O_2 pressure of the blood greatly exceeds the rise in CO_2 pressure. Secondly, in the diffusion of the two gases between the capillaries and the tissues cells, a 20-fold greater diffusion head for O_2 is required than the reverse diffusion head for CO_2. Thus, with the possible exception of the blood in the systemic arteries and pulmonary veins, all the remaining tissues in the body must have a total gas pressure lower than the atmosphere. This is illustrated in figure 1, where all the white areas represent those regions which have a total gas pressure of 1 atmosphere or 760 mm Hg, while the black area represents the tissues with a total gas pressure of approximately 700 mm Hg. This value is a reasonable assumption but will vary from tissue to tissue, depending upon its perfusion rate and rate of metabolism. A gas pocket introduced into the tissues is, therefore, analogous to a gas bubble submerged under water to a depth equivalent to 60 mm Hg.

Any gas which diffuses out of the pocket is carried away by the circulation. Thus, during the life of the gas pocket, this total gas pressure differential is maintained (until the volume is reduced to such small dimensions that the surface tension forces actually increase this differential). This maintained gas pressure difference has several important consequences:

1) A gas pocket continuously decreases in size and must ultimately disappear.

2) During this process of reabsorption (no matter what gas is originally introduced) the gas pocket attains a state of constant composition. That is, the fractional compositions of the gases become and remain constant even though the total gas volume is continuously diminishing.

3) From the above it follows that for each gas present there must be a partial pressure difference between that found at any time in the pocket and the tissues surrounding the pocket. A true equilibrium can never be achieved.

4) Finally, the sum of the partial pressure differences for each gas must equal the total gas

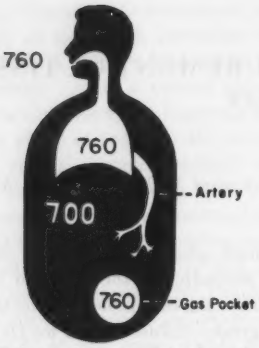

FIG. 1. Schematic representation of the sum of the partial pressures of gases in the body. A gas pocket will remain essentially atmospheric but is surrounded by a considerably lower total gas pressure environment.

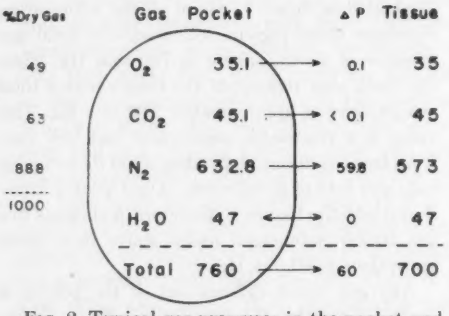

FIG. 2. Typical gas pressures in the pocket and surrounding tissues. For details, see text.

pressure difference between the gas in the pocket and the surrounding tissues.

O_2 Partial Pressure Difference Between Gas Pocket and Tissue. If no true equilibrium exists between the O_2 in the two phases, then we must determine how large this O_2 gradient is in order to make proper corrections for tissue O_2 tension. This can be done theoretically as well as experimentally.

THEORETICAL APPROACH

Two theoretical approaches have been used. In the first case it was assumed that the blood perfusing such a pocket comes into equilibrium with the gases in the pocket. This would be analogous to the gas uptake from an alveolus in the lung. If one assumes that the incoming blood has finite gas tensions, one can apply equations based upon the Fick principle, and with the aid of iterative equations arrive at the O_2, CO_2 and

FIG. 3. Subcutaneous gas pocket in the rat. These pockets can be maintained for months with intermittent refills. The total gas content can be quantitatively aspirated into a syringe and immediately re-introduced for determinations of volume changes with time.

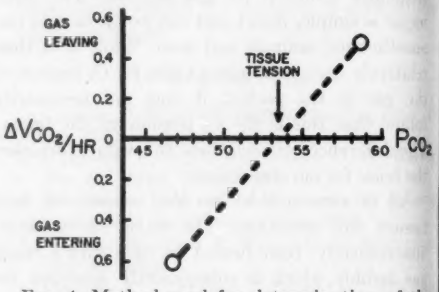

FIG. 4. Method used for determination of the tissue gas tension in an air pocket. (Van Liew, ref. 3). For details, see text.

N_2 pressures which must exist in the gas pocket (1). This method indicates that the O_2 difference would be less than 1 mm Hg. Figure 2 illustrates the compositions found in a typical gas pocket in man. The tissue tensions were assumed, while the gas tensions in the pocket were predicted. The column on the far left indicates the values which one would obtain upon gas analysis of the gas pocket content. It will be noted that the sum of the partial pressure differences must be equal to the total gas pressure difference, and that 99% of this difference is carried by the N_2 gas. This is the least diffusible gas and limits the rate of total gas volume resorption. It is the presence of this rate-limiting gas which allows the more diffusible gases to approach so closely the tissue tension.

Loeschcke (2) has recently employed another theoretical approach, namely that this process of gas resorption is diffusion-limited. By applying the diffusion constants of gases in water, he has calculated the gas pressure gradients. These differ little from those cited above as long as the animal breathes air.

EXPERIMENTAL APPROACH

Van Liew (3) injected various known O_2 mixtures into subcutaneous gas pockets of rats (fig. 3) and determined the rate of O_2 loss as well as the mean O_2 pressure existing at the time. In other cases, he injected O_2 mixtures which had a lower tension than that expected in the tissues and measured the rate of O_2 gain of the pocket as well as the mean O_2 pressure existing in the pocket at that time. When the rate of O_2 loss or gain is plotted against the O_2 pressures existing at the time, one can obtain a linear relationship and interpolate to the O_2 pressure in the gas pocket at which one would predict no O_2 exchange at all. This was assumed to be the O_2 tension of the tissues surrounding the gas pocket. The method of graphing the results is illustrated in figure 4. This particular graph illustrates the results when CO_2 was used as the test gas instead of O_2, but the principle is the same for the procedure testing O_2. Some 32 experiments yielded a calculated gradient of 0.1 mm O_2 when animals breathed air and a 2 mm O_2 gradient when animals breathed pure O_2. Thus, the theoretical and experimental procedures indicate that this method of direct gas analysis of a tissue gas pocket yields an O_2 tension as well as a CO_2 tension which reflects with n 1 mm or less the tension of the gases of the surrounding tissues.

FIG. 5. Blood vessels lining tissues surrounding a subcutaneous air pocket of a rat. Vessels are injected with colloidal mercury sulfide before autopsy. This vascular development took place in less than 2 weeks after initial injection of air. (Tobin, Van Liew and Rahn, ref. 4).

NATURE OF TISSUES SURROUNDING A GAS POCKET

So far, the picture may seem ideal, for by this relatively simple method we measure not only the O_2 but also the CO_2 tension of the tissues surrounding the gas pocket. However, we must now ask two important questions. First, to what extent have the tissues been modified by the introduction of a gaseous foreign body? Second, what particular tissue is being measured by this method; is it the extensive network of the blood vessels, the tissue cells between the vascular supply or an integrated value of all the cells exposed to the gas supply? These questions must be directed toward any method which introduces a foreign body, be it a gas bubble or an oxygen electrode.

MORPHOLOGICAL CHANGES

The histology of the subcutaneous layer of the rat skin have been examined following the introduction of gas (4). Within a few days extensive changes in fibroblasts and collagenous fibers form a well-organized connective tissue wall around the gas pocket. However, the most impressive

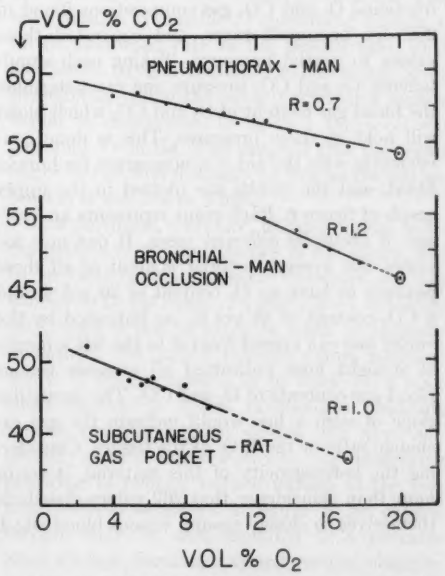

FIG. 6. Calculated simultaneous O_2 and CO_2 content of blood draining 3 types of gas pockets. The assumption is made that the O_2 and CO_2 partial pressures of the gas pockets become identical with those of the blood draining the pocket. Each point on the graph is based on average values reported in the literature. For details see text.

feature is the tremendous growth of the vascular supply. Figure 5 is a whole mount of the lining of such a pocket showing the newly developed vessels. These were filled by the injection of colloidal mercury sulfide into the abdominal aorta before autopsy, and reveal an extensive branching of the venules. One is impressed with the relatively large surface area of venules and capillaries exposed to the gas in contrast to the small area of exposure of arterioles and arteries.

VENOUS BLOOD AND POCKET GAS TENSIONS

In order to determine the possible relationship between the composition of the perfusing blood and the gas tensions in the gas pocket, we proceeded as follows. We made the assumption that the gas tensions of the pocket were essentially the same as those of the venous blood which had previously exchanged with the tissues surrounding the pocket at a particular R.Q. In other words, the air pocket acts like an aerotonometer in which the gas phase comes into equilibrium with venous blood as far as O_2 and CO_2 are concerned.

From the literature (5–7) we assembled the fractional O_2 and CO_2 gas compositions found in 262 dry pneumothoraces, and converted these values to partial pressures. Taking each simultaneous O_2 and CO_2 pressure one can determine the blood gas content of O_2 and CO_2 which blood will hold at these pressures. This is done conveniently with the aid of a nomogram for human blood, and the results are plotted in the upper graph of figure 6. Each point represents an average of about 20 different cases. If one now assumes the average arterial content of all these patients to have an O_2 content of 20 vol % and a CO_2 content of 48 vol % (as indicated by the circle) one can extend from it to the left a family of straight lines indicating all possible venous blood gas contents of O_2 and CO_2. The particular slope of such a line would indicate the gas exchange ratio or the R.Q. of the tissues. Considering the heterogeneity of this material, it seems more than coincidence that 262 values distribute themselves to closely around venous blood which must have exchanged with the tissues at an R.Q. of 0.7.

A similar analysis was made of 35 gas analyses obtained by Loewy and von Schrotter (8) after occlusion of one bronchus in man. These data are shown in the middle graph of figure 6.

Finally, 58 gas samples taken from the subcutaneous pockets of rats were analyzed with the aid of a blood nomogram for rat blood. These findings are shown in the lower graph of figure 6.

From these findings, it would appear as if the gas tensions found in pockets are very similar to those which one would expect to find in venous blood. The wide variations in O_2 tensions which can be found among various individuals would then indicate different perfusion rates.

SUMMARY

The steady state gas pocket is a convenient tool for estimating the Po_2 and Pco_2 of the surrounding tissues. No correction needs to be applied for the O_2 gradient when air is breathed.

However, the tissues surrounding a gas pocket cannot be considered normal since morphological changes in response to the foregin material have occurred. A great increase in the vascular supply is probably responsible for the fact that the gas tensions in such a pocket are nearly identical with those which one would expect in venous blood. Differences in gas compositions among various individuals can be assumed to be related to differences in blood perfusion rates.

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OXYGEN TENSION IN MAMMALIAN BRAIN

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BECAUSE of the high rate of oxygen consumption of the brain, it must be supplied with a rich vascular bed, if those points midway between adjacent capillaries are not to be in a state of permanent anoxia. The subject of this brief sketch is to describe what oxygen tensions are found in the mammalian cerebral cortex, and to discuss the possibility that the oxygen tension may be low enough in the microscopic regions between capillaries, under certain conditions, to give rise to local metabolic deficiencies. The illustrations will be drawn from some work that was done by us at the Johnson Foundation during the last war.

The oxygen consumption of whole mammalian brain, as measured by various investigators (1–3), is of the order of 2,500 mm^3/gm hr. (wet weight), which is 10 or so times as great as that of mammalian peripheral nerve. Probably the largest single cause of this high metabolic rate lies in the work required for the active transport of sodium ions out of all parts of the cortical neurons against a combined voltage and concentration gradient. There is a large amount of total neuronal membrane area in the cortex, especially due to dendrites and to fine axon terminations, and a simple calculation suggests that a sizeable fraction of all the energy available from oxidation of substrates would be needed to keep sodium pumped out through such membranes, even if the pumping were done at 100% efficiency.

Figure 1 shows how rapidly the cortex uses up its supply of dissolved oxygen if the circulation is occluded locally (4). This record was obtained by placing an oxygen cathode gently at first against a small vein on the surface of the cortex of a lightly anaesthetized cat. The oxygen tension of the venous blood of the pial circulation had been measured just previously with a recessed oxygen cathode, and had been found to be approximately 25 mm Hg. At the first break in the record, the electrode was made to press hard enough to occlude the cortical circulation in the vicinity of the tip, causing the cortex there to draw on its local supply of dissolved oxygen. In less than a second the oxygen tension fell in nearly linear fashion from the venous value to a low level, and then ceased to fall further. The horizontal line drawn in from the right at the end of the record represents the zero of electrode current, or the level of zero oxygen tension.[1] This curve is the counterpart for the living cortex of the oxygen consumption curves that Dr. Chance reported earlier for his isolated enzyme systems. It proves that the cortex has a very small reserve of dissolved oxygen, should the circulation fail completely. It also demonstrates that the cortex, in common with most tissues, is capable of consuming oxygen at an undiminished rate as the tension falls, until the tissue oxygen tension reaches a low level, presumably where the oxygen activating system begins to unsaturate. In figure 1 this level was less than 5 mm Hg. This suggests that the cortex ought to function normally as long as the oxygen tension is well above 5 mm.

It is pertinent, therefore, to examine the existing pattern of oxygen tensions in the cortex, and to see whether the tensions anywhere are low enough to be the cause of a diminished metabolic rate. Since it can be made as small in diameter as desired, the oxygen cathode allows measurements of oxygen tension to be made on a scale small enough to reveal microscopic gradients around individual blood vessels. We have employed oxygen microelectrodes for measuring gradients of relative oxygen tension at the cortical surface, as shown in figure 2. An oxygen cathode may be seen mounted in a movable glass window, forming a closed cortical chamber

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[1] Curves as linear as this are somewhat difficult to obtain on the cortex, probably because of diffusion transients. They seem to be more easily obtainable under conditions of marked vasodilation (e.g., during administration of 95% O_2-5% CO_2), when gradients of tissue oxygen tension around capillaries would be minimized.

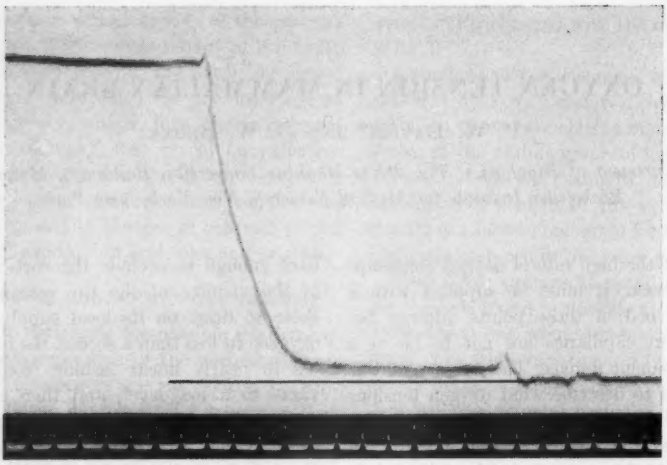

FIG. 1. Record of O_2 tension at surface of cortex during local occlusion of the circulation. Obtained with a 27μ Pt open type electrode. OD of glass insulating sheath at tip, 150μ. Electrode on a small pial vein. O_2 tension of vein before occlusion, approximately 25 mm. Electrode on vein during occlusion. A line is drawn through the horizontal part of the curve after the break at the extreme right, representing the zero of electrode current, or zero oxygen tension. Minor time intervals, 1/5 sec.

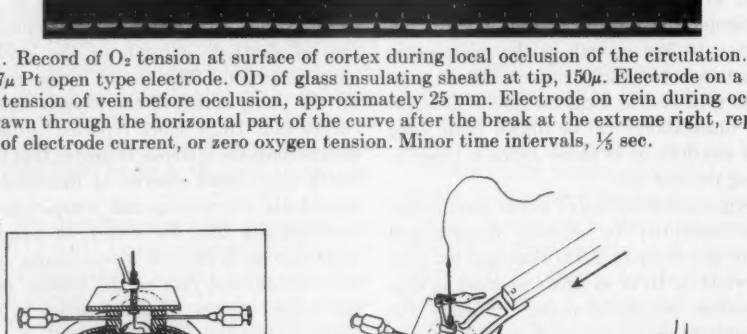

FIG. 2. Diagram of oxygen cathode mounted in a closed cortical chamber for measuring surface Po_2. Side tubes are normally closed.

which nevertheless allows the electrode to be positioned at any desired cortical spot. The closed chamber prevented pulsations of the exposed cortex which would have greatly hampered the measurements. With the aid of a dissecting microscope the electrode was placed at various positions relative to individual pial blood vessels, and the results are shown in figure 3.

This diagram shows the relative oxygen tension measured with a 14 μ electrode at points along a line crossing three pial blood vessels. It can be seen that in spite of the small distances between the vessels—not more than 150 μ—there are steep downward gradients of oxygen tension between them along which the oxygen diffuses from the vessels into the tissue. We thus have objective evidence of steep diffusion gradients in a tissue of high metabolic rate. It should also be noticed that a venule, perhaps contrary to orthodox concepts, is capable of supplying oxygen to its local environment.

These measurements were made with an open type electrode (5). Such an electrode is affected by variations in diffusion coefficient as well as oxygen tension, and its readings against blood vessels with the blood flowing inside may well give spuriously high values in the immediate vicinity of the vessels. A more reliable type of electrode for making absolute measurements of oxygen tension is the recessed electrode, with an extension of the glass insulating sheath beyond the metallic tip, forming a small chamber so that the electrode reaction always takes place in a medium of unvarying diffusion coefficient. The electrode is operated intermittently, and between readings the oxygen in the recess comes into equilibrium with that of the tissue at its orifice. This electrode is thus capable of giving readings

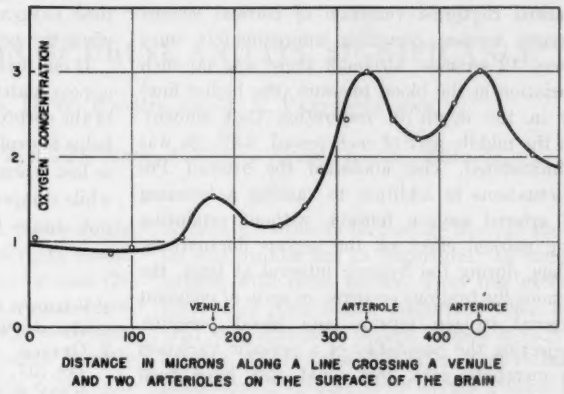

FIG. 3. Relative O_2 tension at cortical surface in neighborhood of three pial blood vessels of a cat under moderate Nembutal anaesthesia and breathing air. Positions and diameters of vessels are indicated by circles drawn to scale on the horizontal axis. Obtained with a 14μ Sb open type electrode. OD of glass insulating sheath at tip, 27μ. Horizontal portion of electrode (see fig. 2) was drawn down to approximately 4μ to give it flexibility. This precluded any possibility of compressing the circulation locally, which would have produced abnormally low values of P_{O_2}.

independent of variations in external diffusion coefficient. Such an electrode has been used for making oxygen tension measurements at various locations on the cortical surface as far away from pial vessels as possible. The diameter of the orifice was about 30 μ with a recess length of 0.38 mm. The electrode was calibrated *in situ* against small diameter pial arteries, which generally have an oxygen tension close to 100 mm for a cat breathing air. It was found that when great care was taken not to compress the cortex in the slightest degree, the values of oxygen tension at 15 of these remote points in 3 cats lightly anaesthetized with Nembutal varied from about 2 mm to about 10 mm, and were distributed more or less equally in this range, with the exception of one value, 17.5 mm. Doubtless the variation was due to differences in the nearness of underlying capillaries, which could not be clearly distinguished with the dissecting microscope. Thus, under our experimental conditions, which are probably not far different from normal in respect to blood flow and metabolism,[2] many cortical locations have oxygen tension values nearly low enough to cause metabolic impairment.

Thus it seems likely that the cortex is on the verge of oxygen insufficiency, even in its normal state, and this raises the question of compensating mechanisms. It is well known (6) that the usual nervous and chemical mechanisms regulating the circulation are operative in the cortex, and it is sometimes stressed that in the cortex these mechanisms act more to produce vasodilation than vasoconstriction. That the vasomotor system is sometimes capable of producing rather pronounced variations in cortical oxygen tension, however, is shown in figure 4. The upper record was taken with an oxygen cathode against a pial artery of a cat that had apparently developed a rhythmic variation in vasomotor tone. In the lower record the electrode recorded oxygen tension of a pial vein. Looking especially at the

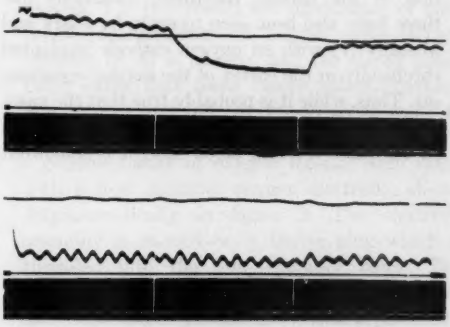

FIG. 4. O_2 tension measured with a 25 μ Pt open type electrode on a 60 μ pial artery (*upper record*) and on an 85 μ pial vein (*lower record*) of a cat under Nembutal anaesthesia (body temperature, 33°C). The thin unbroken upper line in each record, showing no oscillations, is blood pressure recorded from the femoral artery (average blood pressure, 83 mm Hg). O_2 (8.3%) was administered between vertical white lines in each time base. Horizontal black line drawn in just above time base represents zero O_2 tension. Minor time intervals, $\frac{1}{5}$ min.

[2] According to Schmidt, Kety and Pennes (1, 3) the cerebral blood flow and oxygen consumption of monkeys lightly anaesthetized with Nembutal are about the same as in unanaesthetized human subjects.

lower record, it can be seen that there was a marked rhythmic variation of cortical venous oxygen tension occurring approximately once every 12 seconds, although there was no such variation in the blood pressure (the higher line) or in the depth of respiration (not shown). In the middle part of each record, 8.3% O_2 was administered. This abolished the arterial Po_2 fluctuations in addition to causing a lowering of arterial oxygen tension, without producing any marked effect on the venous fluctuations. Thus, during the hypoxic interval at least, the venous fluctuations occurred in spite of constant arterial oxygen tension and blood pressure. Rejecting the possibility of a periodic variation in metabolic rate, this could only have been due to alternate vasoconstriction and vasodilation in the cortex. Rhythmic variations like these have also been seen recently by Clark and Misrahy (7) with an oxygen cathode implanted chronically in the cortex of the normal conscious cat. Thus, while it is probably true that the vasomotor system operates normally to maintain an adequate cerebral blood flow, it is somewhat disconcerting to see that such large shifts in cortical oxygen tension can occur spontaneously, when the normal margin of safety is so narrow.

If our findings can be accepted as representing approximately normal conditions, the nearness of the cortex to a state of anoxia, at least locally, helps to explain the ease with which consciouness is lost when the oxygen supply is reduced, and while compensatory mechanisms exist, they may not always be entirely effective.

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OXYGEN TENSION OF URINE AND ITS SIGNIFICANCE

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The large blood flow per gram tissue weight and the high oxygen saturation of renal venous blood, 85% in dogs (1) and higher in man (2), have been taken as an indication that blood within the kidney has a high oxygen tension. In the theory of renal hemodynamics recently suggested by Pappenheimer and Kinter (3) it is postulated that red blood cells are partially separated from plasma within the kidney by a process of plasma skimming, and that a cell-rich component of the blood is diverted through a short circulation bypassing the peritubular capillaries, leaving a cell-poor moiety of the blood to perfuse the renal tubules. It would follow that the peritubular capillary blood has a lower oxygen tension than renal venous blood and that the tubules are not supplied with as much oxygen as has been generally assumed in the past. In the present study the oxygen tension of urine was examined as an indicator of the peritubular capillary blood tension.

Simultaneous measurements of the oxygen tensions in renal pelvic urine and renal venous blood and of the clearances of creatinine and Diodrast I^{131} were made in dogs under Nembutal anesthesia. From a catheter placed fluoroscopically, renal venous blood was obtained for the determination of renal vein oxygen tensions and Diodrast extraction ratios. Urine obtained by cannulating the ureter close to the kidney was used for all oxygen measurements because at least two factors conspire to make the oxygen tension of bladder urine different from that of renal pelvic urine. First, the occasional presence in urine of powerful reducing substances capable of reacting with dissolved oxygen noted by Berthelot (4) and Krogh (5), requires that this source of error be eliminated. This was achieved by requiring that an acceptable measurement be obtained from a sample whose oxygen tenson was stable over a period of minutes. When reducing substances such as ascorbic acid are present the oxygen tension falls rapidly to zero with time. Second, the urine in its passage through the ureter and bladder has an opportunity to exchange oxygen with these tissues. That this exchange may be of considerable magnitude when measured as a change in oxygen tension is shown in figure 1. At low urine flow rates, large errors are introduced if bladder urine oxygen tension is to be considered a measure of renal pelvic urine tension. For this reason the significance of published measurements on human urine oxygen tensions (5, 6) is unclear, and the strong possibility exists that these values reflect considerable oxygen exchange by the urine en route to the site of collection.

Measurements of oxygen tension were made with a flow chamber oxygen electrode, shown diagrammatically in figure 2. The electrode assembly is carried on a Lucite plug which is threaded into the flow chamber block. The platinum electrode is sealed into the end of a soft glass tube and so ground down that a plane surface is obtained which presents only a cross-sectional area of the 2-mil diameter platinum wire. The exposed surface of a heavy silver wire, also carried on the electrode assembly, is chlorided and serves as the anode for the electrode unit. Electrical contract to the platinum electrode is made by means of a mercury pool in the glass tube.

A number of difficulties are met when the oxygen cathode is used to determine the tensions of biological fluids, particularly whole blood. These difficulties are due to the fact that for quantitative use of the oxygen cathode the diffusion shell at the platinum surface must be mechanically stable and must occur in a fluid whose oxygen solubility coefficient is the same as that of the fluid against which the electrode was calibrated. Also, the presence of protein apparently initiates a loss of effective surface area which manifests itself as a downward drift in the calibration curve. These difficulties have been overcome by the interposition of a small

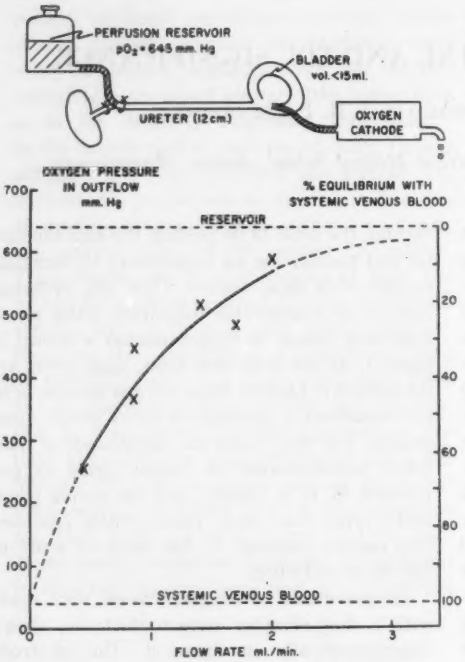

FIG. 1. Diffusion of O_2 from urinary tract. O_2 tension of Ringer's solution after perfusion of ureter and bladder in a dog (15 kg). *Above*, schematic diagram of perfusion system. Bladder permitted to drain continuously. *Below*, O_2 tension of perfusate as a function of flow rate; *X*'s are experimental points for effluent O_2 tension; *dashed line* is the initial O_2 tension in reservoir.

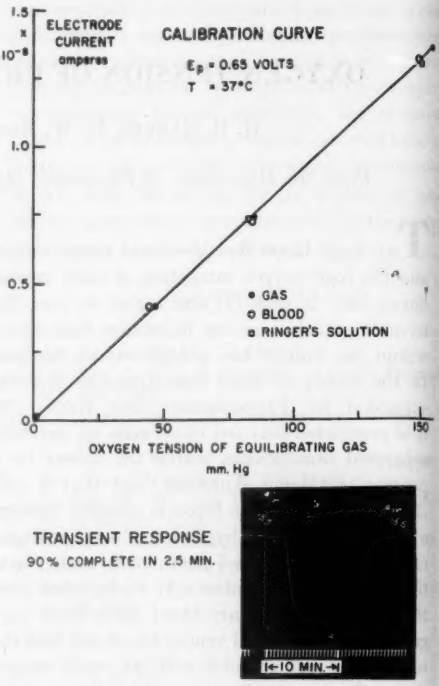

FIG. 3. *Above*, calibration curve for flow-chamber oxygen electrode. Note that experimental points for gas, whole blood and Ringer's solution at a given O_2 tension have the same current reading in spite of their large differences in O_2 content. *Below*, a kymograph recording shows the response time to a step change from air to tank nitrogen and back again at 37°C.

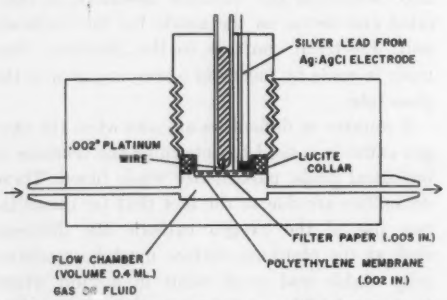

FIG. 2. Diagrammatic cross-section of electrode assembly in flow-chamber block. A separate electrolyte compartment at surface of the electrode, maintained in the filter paper matrix, is covered by a polyethylene membrane. Polyethylene is permeable to oxygen but does not permit significant exchange of other aqueous components. Membrane and filter paper are assembled while electrode assembly is under saline to prevent enclosure of air bubbles.

compartment of electrolyte (see fig. 2), Ringer's solution or 0.1 N KCl, held in a single thickness of filter paper and covered by a polyethylene membrane permeable to the gaseous components of aqueous solutions. The membrane and filter paper are held in place by a snug-fitting Lucite collar. In this manner the greater portion of the diffusion shell is held within the stationary membrane, making the readings insensitive to flow-rate and to changes in the composition of the solution measured. Figure 3 shows a typical calibration curve. It should be noted that points for gas, whole blood and Ringer's solution fall along the same line; not shown but also falling on this line are points for 20% sucrose solutions. The inset figure in figure 3 indicates the time course for a step-change in oxygen tension. Depicted here is the change from air to tank nitrogen and back again at 37°C. The slow re-

sponse is a definite limitation, but for the steady state measurements reported here this response time was adequate.

The use of two electrodes permitted the urine tension to be recorded continuously while arterial and renal venous blood tensions were alternately sampled. These tensions are shown in figure 4 for kidneys in which the measured blood flows, glomerular filtration rates and oxygen consumptions were normal. It may be seen that the oxygen tension in the renal vein is well above the comparable urine tension, and that as the tension in the renal vein is raised, the difference increases. The latter property makes it unlikely that the magnitude of the difference can be accounted for by a gradient of oxygen tension existing across the renal tubule cells in passing from the peritubular capillary plexus to the tubular urine. This conclusion is further supported by calculations of the order of magnitude expected for such a gradient based on the diffusion equations developed by Krogh (7) and extended by A. V. Hill (8). The results of such calculations are shown in figure 5; even with improbably large values for the average non-vascular oxygen consumption and tubular dimensions (9), the gradient so calculated amounts to less than 5 mm Hg. Inasmuch as the collecting ducts penetrate the same peritubular capillary plexus as surrounds the nephron tubule cells, it is likely that the urinary oxygen tension approaches that found in the peritubular capillary blood. For these reasons it is suggested that peritubular capillary blood and renal venous blood are distinct with respect to their oxygen tensions as postulated by the cell-separation theory.

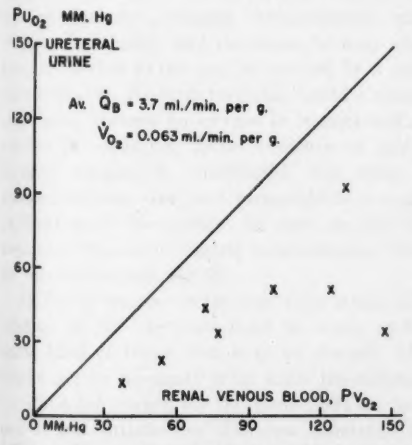

FIG. 4. Comparison of oxygen tension in ureteral urine with that obtaining simultaneously in renal venous blood. Renal oxygen consumptions ($\dot{V}o_2$) and renal blood flow ($\dot{Q}_B$) were measured at each point; average figures for series are indicated. Experiments on anesthetized dogs breathing O_2 mixtures.

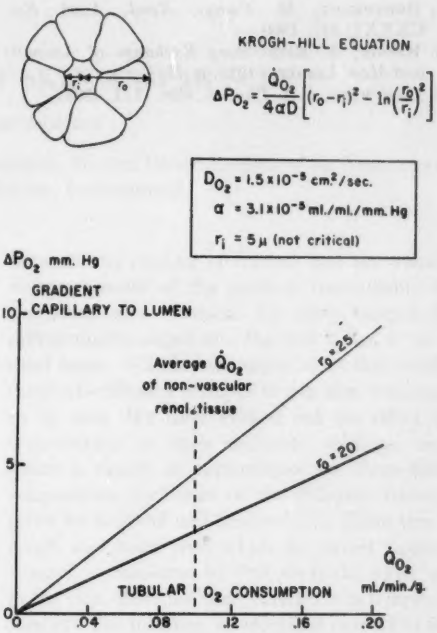

FIG. 5. Calculation of average O_2 tension gradient across renal tubule cells. *Above*, Krogh-Hill equation used for this calculation is given, where r_o is the outside tubule radius; r_i, the inside tubule radius; Do_2, the diffusion coefficient; and α, the solubility of oxygen in tissue. *Below*, solution of equation for gradient across the tubule cells (ΔPo_2) is given as a function of the oxygen consumption of the kidney (Qo_2 ml/min/gm). The value for the average Qo_2 of non-vascular renal tissue is purposely chosen large in order to obtain an order of magnitude for the maximal gradient to be expected.

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OXYGEN TENSION OF SKIN AND MUSCLE

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I SHALL confine my remarks largely to studies we have made on changes in oxygen tension in human skin and muscle. In 1947 Martin Larrabee and Philip W. Davies, when they were in the Johnson Foundation of the University of Pennsylvania, equipped us for the work (1, 2). Dr. Orville Horwitz and I began the studies; Dr. Raymond Penneys and others have joined us in the meantime, and most of the work has been published (3–15). Our interests are primarily in peripheral vascular diseases, and we were glad to try the amperometric method with the platinum electrode, to learn something about oxygen in human normal and ischemic extremities.

STUDIES ON SKIN

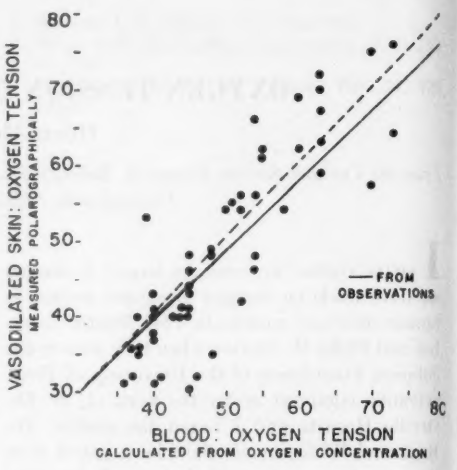
The first work was on skin. Larrabee had demonstrated with the open-tip platinum electrode that electrode current, and therefore presumably oxygen tension, of the skin of the human forearm increases conspicuously when oxygen is inhaled, and decreases to zero when the circulation to the arm is arrested by a pressure-cuff (16). We confirmed this, found a reasonably good plateau for oxygen in current-voltage curves in solutions, found evidence of higher oxygen tension in vasodilated skin than in vasoconstricted skin, and proceeded to a rough calibration of the method for skin, so that the results of the amperometric measurements could be reported in mm Hg (3).

As far as we are aware, any even rough calibration of the electrode must be made in the same kind of tissue that is to be studied. This might not be necessary if we knew the diffusion constant for oxygen for the tissue, but we have not found satisfactory diffusion constants for human skin or muscle. The electrode consisted of the exposed tip of a 0.2-mm platinum wire. We used −0.6 v. with 10-sec. closures every 2 or more minutes. Small bits of dead full-thickness human skin were placed over open-tip platinum electrodes in solutions of known oxygen tension, and a straight-line relationship between the reading of current and the known oxygen tension of the solution (presumably of the skin) was obtained. To allow oxygen to diffuse undiminished into the skin it had to be a dead tissue. Whether the use of dead skin would duplicate diffusion distance of live skin remained to be seen. We then worked out the effect of temperature on such electrode readings, and found it closely to approximate the theoretical temperature coefficient of the diffusion current given by Kolthoff and Lingane (17). From this a graph was made with which to correct oxygen tension, as measured by this electrode, when, as in the skin, there are wide variations in temperature (4). For instance, an electrical current of 80 read at 30°C, indicates an oxygen tension of X, whereas one of about 60 read at 10°C also indicates an oxygen tension of X. On analyses of this method of calibration we found an average error of about 25% for any electrode repeatedly inserted in skin. This is a wide error, but with multiple electrodes it diminishes, and we have on occasion presented semi-quantitative data, in mm Hg, in this way.

Those of us who are interested in peripheral vascular diseases are constantly aware of the low utilization of oxygen by skin, and of the high blood flow through skin during cutaneous vasodilatation. We had every reason to believe, therefore, that during vasodilatation the oxygen tension of skin should approximate the oxygen tension of arterial blood. So, when a mean value of 90 mm Hg was found for the oxygen tension of vasodilated skin, when using the calibrated electrode, we considered the method of calibration a promising one. Perhaps within skin the factor of distance of diffusion of the gas to the electrode varies little, or if it varies, the factor is not a critical one. Unsuccessful attempts were made to evoke this variable by distending capillaries by pressure on veins. Pneumatic cuff pressure up to 30 mm greatly increased the depth of color in skin, and no change in current readings resulted. Judging from skin temperature, cutaneous blood flow was not altered (18). We were further en-

couraged in the use of the electrode in human skin by finding reductions in oxygen of skin relating to decreases in circulation caused by vasoconstriction and by arterial occlusion such as that produced by arteriosclerosis (4).

We then studied concomitantly the oxygen tension of reflexly vasodilated skin and the percentage of saturation of arterial blood by oximetry, while varying the saturation over the safe range of 70–100% saturation. The values for changing oxygen tension of skin, when plotted against the corresponding values for saturation of blood, fell closely upon the conventional dissociation curve for oxyhemoglobin in red cells (8). A comparison of tensions by the two methods is shown in the figure. The dotted line is that of identity between oxygen tension of vasodilated skin and of arterial blood. The solid line is that obtained experimentally. The scatter is about that expected from a variation in pH of ±0.2.



This was further evidence that the amperometric method is suitable for human skin. It also laid the groundwork for studies of changes in oxygen tension of arterial blood in patients (5). Since then we have made a number of physiological and clinical studies on oxygen tension of the skin of the extremities of normal subjects and of patients having peripheral vascular disease (4, 9). These, and studies designed to evaluate the effect of vasodilator and vasoconstrictor drugs (14, 19) and therapeutic procedures will not be reported here (7, 10, 15). Usually no calibration of the electrodes has been necessary; directional and percentile changes have sufficed. Multiple electrodes, rather than a single electrode, lend conviction to the work.

One might ask whether this method is in some cases measuring oxygen tension of capillary blood rather than of tissue fluid. I think not. In measuring changes in oxygen tension in skin induced by sudden changes in capillary oxygen, as by removing an arterial tourniquet from the arm, the change in oxygen tension is delayed, showing time taken for diffusion of oxygen from the capillaries to the electrode in the tissue.

STUDIES ON HUMAN SKELETAL MUSCLE

These have been somewhat more difficult than those in skin. As in skin, we obtained straight-line curves for oxygen tension in dead muscle, but we are less sure of using the data for calibration, and we have confined the studies to measurements of changes in oxygen tension of muscle. We at first used much the same electrode as had been used in skin; that is, a small open-tip electrode, having some 0.05 mm² of surface area. With this small electrode, different insertions into resting skeletal muscle yielded very different results. When a larger surface of electrode was used, the results of repeated insertions were much more uniform (12). Using the larger electrodes in the muscle of the forearm, Dr. H. E. Holling of London and I found the oxygen tension to decrease when the arm was passively elevated, and to approach zero when the circulation to the arm was arrested by a pressure-cuff (20). Oxygen breathing resulted in much less increase in oxygen tension in muscle than in skin. The several drugs studied have not changed the oxygen tension of resting human muscle, as we have measured it (19). In the rabbit, when a leg was chilled the oxygen tension of leg muscle usually decreased a little, and oxygen inhalation (during chilling) restored the tension of oxygen to or above the level in warm muscle (11).

Paul Gabel in Rochester, New York, found, in dogs, that adrenaline introduced intra-arterially or intravenously in concentrations sufficient to reduce the oxygen tension of skin increases the oxygen tension of resting muscle (21). Wolferth *et al.* have, in dogs, studied the effect of moderate hemorrhagic shock on the oxygen tension of muscle. The oxygen tension of muscle decreased; that of liver did not (22).

John Sayen and his co-workers have for several years used the platinum electrode in the myocardium of the dog. They found that adrenaline increases myocardial oxygen tension, as does the

inhalation of oxygen, and ligating a coronary artery decreases oxygen tension. With coronary ligation they found that the ventricle uses most of its oxygen within a period of some 30 seconds. They have shown interesting correlations of oxygen tension and epicardial electrocardiographic data (23, 24, 25).

SUMMARY

In skin and in muscle the open-tip platinum electrode has the advantages of ease of insertion, small size and rapidity of response. It has the disadvantage of difficulty of calibration. Work on oxygen tension in tissue would be improved by a more calibratible electrode which loses none of the advantages of the open-tip electrode.

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DISCUSSION

L. C. Clark, Jr., M. G. Larrabee, A. A. Pandazi, R. H. Shepard and R. R. Sonnenschein

Dr. Comroe: Dr. Chance, do you have further information on the effect upon oxygen requirements of feeding, starvation, uncoupling agents and hormones such as thyroxin?

Dr. Chance: This question can be answered qualitatively by physical argument and more precisely as indicated by figures 17 and 18. Quantitatively, it is reasonable to believe that the oxygen requirements would decrease with increasing starvation of the biological system. On the other hand, the oxygen requirement would increase with feeding to a plateau value at which no further increase of oxygen requirement would occur. The effect of addition of ADP, which is similar in some respects to the addition of uncoupling agents and hormones that act as uncoupling agents, has been studied experimentally, and table 3 already shows that only a slight increase of the oxygen requirements would be observed. On a quantitative basis, the analogue computer representation of the properties of the respiratory chain permits a study of the oxygen requirement for such a system over a much wider range of values than is possible experimentally. The results of such study are indicated in figures 17 and 18. Figure 17 indicates the effect of increasing the substrate concentration (designated by *a* on the abscissa of the graph). It is seen that there is a roughly hyperbolic relationship between the substrate concentration and the oxygen requirement (indicated by 'K_m' along

the ordinate). For the more complicated case in which the amount of a particular enzyme of the respiratory chain is varied, due to the effect of a specific inhibitor or uncoupling agent, we have obtained solutions shown in figure 18 in which the variation of the middle enzyme of a sequence of three is carried out and the effect upon the oxygen requirement 'K_m' is indicated. These effects depend somewhat upon the amount of substrate present; thus two curves are given, one for a large and one for a small amount of substrate. At the lower substrate concentration, it is clear that a 5-fold variation of the concentration of the middle enzyme of the chain would cause a nearly insignificant change of the oxygen requirement. Thus it is seen that the oxygen requirement is a relatively stable characteristic of the respiratory system and that considerable variation of the nature and activity of the components can occur without a major variation in this property.

DR. SHEPARD: How do you know that the action of the respiratory chain is essentially irreversible?

DR. CHANCE: This is a most important question in considerations of critical oxygen tension. Although the oxidase has not yet been titrated with oxygen, we know from the following experiment that its reaction with oxygen is essentially irreversible. Whole cells or cytochrome components extracted therefrom are allowed to respire until they become anaerobic due to their own respiration. Then the strong reducing agent, sodium hydrosulfite, is added. This chemical would reduce any remaining ferric iron-porphyrin compounds to the ferrous state. No increase in the reduction is caused by hydrosulfite. Thus the oxidase iron is completely reduced by an irreversible reaction with oxygen. That hydrosulfite acts as a specific reductant of the oxidase and not as an oxygen remover has been shown by unpublished experiments in collaboration with Dr. Lucile Smith in which the purified oxidase was titrated with hydrosulfite under anaerobic conditions.

DR. LARRABEE: How do you define resting state of metabolism?

DR. CHANCE: We define resting state of metabolism as that which results from the lack of phosphate or phosphate acceptor in a system supplied adequately with oxygen and substrate. In living tissue we use as an example a resting muscle where lack of phosphate or phosphate acceptor causes a 10-fold decrease of respiratory activity in mammalian muscle and perhaps a 100-fold decrease in insect flight muscle.

DR. COMROE: When one implants an oxygen electrode in tissue and measures oxygen tension, what tissue does the electrode 'see'?

DR. MONTGOMERY: Much of the discussion has implied that an electrode is in a position near, or far from, a capillary. That is true of Dr. Davies' micro-electrodes on the cat's brain. However, most electrodes used in tissues are large ones which damage and perhaps compress adjacent capillaries and cells. Some compression may be an advantage; this may act as a useful coating for the electrode. It may in part account for the stability of the electrode, at least in skin. There has been some confusion about what oxygen we are measuring and whether it is worth measuring. Aren't we measuring intercellular oxygen tension, and isn't that what the cell depends on? There are gradients of oxygen in flowing capillaries, and the cell is drawing oxygen from these: we are measuring the changes in the mean oxygen of the intercellular spaces.

DR. CHANCE: With reference to Dr. Daves' comments on the definition of tissue oxygen requirements, the physiologist might state that the tissue oxygen requirement is a pattern due to the interaction of oxygen supply and demand. It is also possible to define it at the molecular level, in terms of the oxidation state of the respiratory carriers inside the particular cell. On this basis, one could evaluate critically the hypothesis that Dr. Davies has advanced that cells that lie distant from venules and arterioles in brain tissue are at the 'brink of anoxia.' Actually, an experimental test of the oxidation state of cytochromes in living tissue is of utmost importance in order to provide a basis for the interpretation of impairment of cerebral function caused by small changes of arterial oxygen tension.

DR. CLARK: I was very happy to learn that Dr. Davies has observed waves similar, if not identical to those I described in a paper yesterday (CLARK, L. C. AND G. MISRAHY. *Fed. Proc.* 16: 22, 1957). These periodic fluctuations, occurring at the rate of 6 to 10 per minute, are always observed in chronically implanted electrodes and sometimes have a magnitude as high as ±30% of the mean cathode oxygen availability. Since both the cathode and the brain cells are consuming oxygen, the cells may be just as subject to the stirring effect as the cathode. If the parallelism is roughly quantitative, as it appears to be, then cerebral cells obtain oxygen in waves of undetermined origin.

DR. COMROE: How small can one make oxygen cathodes?

DR. CLARK: We have been using metal cathodes about 10 μ in diameter in measurement of oxygen availability in cochlear fluids. These were gold-plated indium filled Dowben-Rose electrodes. The smallest membrane-covered oxygen electrode I have made has the external diameter of a 20-gauge needle.

DR. COMROE: I think it might be helpful if each of you would discuss some of the problems en-

countered in the use of solid O_2 electrodes and possible solutions of these.

DR. PANDAZI: It seems that much of the difficulty one encounters when using solid cathode polarography for estimating physically dissolved oxygen is associated with polarization of the cathode. Polarization may occur as the result of at least two processes: the first is related to the application of the necessary electrical potential, and the second to the migration of material from the medium to the cathode surface. In simple solutions such as isotonic sodium chloride, electrical polarization dissipates exponentially with time after voltage is turned off. We have obtained reproducible polarograms and calibration curves in saline with plain, saline-filled recessed or colloidion-coated platinum cathodes which were polarized to the same extent before each reading. However, it appears that there exists a direct relationship between the complexity of the media and the time necessary for complete depolarization. We believe that the difficulty associated with obtaining reproducible results in plasma and whole blood is related to the additive effects of varying amounts of residual polarization. In order to take advantage of the characteristics found when using saline, we constructed a recessed saline-filled cathode. Polarograms were obtained by increasing the voltage at a fixed rate (0.40 v/min.) over the range 0.0–1.0 v. They were reproducible at a given oxygen tension and temperature and their magnitude in the range 0.45–0.8 v. is a linear function of oxygen tension. This type of electrode and circuitry has the advantages of *1*) possessing a fixed diffusion layer (hence almost insensitive to external movement); *2*) not being contaminated by environment being tested; *3*) being minimally affected by electrical polarization and *4*) capable of being made very small. At the present time our efforts are being directed toward testing the characteristics of this type of electrode with the hope of ultimately adapting it for use in single cells.

DR. CLARK: The electrode described by Dr. Pandazi has possibilities for being developed for the measurement of P_{CO_2} by using a recessed micro pH electrode as the detector.

DR. SHEPHARD: In most cases it is not possible to calibrate electrodes in the same tissue in which measurements are to be made. Such a procedure is always inconvenient and is often self-defeating because equilibration of the tissue changes the properties one wishes to hold constant. It seems likely, therefore, that accurate estimation of absolute values of P_{O_2} in tissues will require that the electrode have the same calibration in tissues as in simple saline solutions. Three mechanisms can cause the calibration in tissue to differ from that in saline: *1*) 'Poisoning'—the change in calibration which results from contamination of the electrode surface by materials from tissue; *2*) 'solute effect'—the change in calibration which results from change in the diffusion characteristics of the pathway across which oxygen moves to the electrode. Such a change can be produced by a change of concentration of almost any solute in the pathway; *3*) 'movement artifact'—the change in calibration which results from a change in the dimensions of the diffusion pathway owing to movement of the electrode or of the tissue. Change in calibration with movement implies that a significant proportion of the diffusion pathway lies in the tissue and that calibration will also be altered by changes in the tissue (e.g. solute effect). Obviously, all of these difficulties could be overcome if the electrode could be covered with a membrane which is impermeable to substances which produce the 'poisoning' and 'solute' effects and if virtually all of the diffusion gradient between tissue and electrode surface could be confined to this membrane. Dr. Clark's development of the enclosed electrode permits use of a wide variety of covering materials. So far, none of the materials tried seems satisfactory in all respects. Polyethylene membranes seem capable of preventing the 'poisoning' and 'solute' effects, and, in fluid tissues, such as blood, movement artifact can be minimized by rapid stirring. However, movement artifact persists in still tissues. This problem might be solved by interposing a barrier to the diffusion of oxygen between the membrane and the surface of the electrode. If oxygen could diffuse through the membrane much more rapidly than through the barrier, a smaller proportion of the diffusion gradient between tissue and electrode would exist in the membrane. An even smaller proportion of the gradient should exist in the tissue, minimizing the movement artifact.

DR. CLARK: Don't the difficulties encountered in the biological application of solid polarograph cathodes come down mainly to two factors? The first is that the metal becomes contaminated, or 'poisoned' by various substances. This problem has been solved by the membrane electrode (Clark, L. C. *Trans. Am. Soc. Art. Int. Org.* 2: 41, 1956) which utilizes a water-impermeable membrane. This electrode is independent of the ionic composition of the fluid and can even be used to measure oxygen in hydrochloric acid, oil, or gas. The second difficulty is that the cathode consumes oxygen at a rate exceeding that at which oxygen can diffuse through tissues or liquids. This has been partially solved by the protecting membrane which, in itself, lowers the oxygen consumption of the cathode but additional means, such as a porous barrier between the cathode and the membrane are necessary to eliminate this problem (the stirring effect). As yet I know of no satisfactory

method for lowering the oxygen consumption of a bare metal cathode.

Those of you who are using the membrane oxygen electrode will be interested in the properties of a new Teflon film. Samples can be obtained from the Dilectrix Corporation, Farmingdale, Long Island, New York.

DR. SONNENSCHEIN: We have tried various combinations of alternate reversed polarizations of the platinum electrode and of opening and closing of the circuit, based on the suggestions of Olson and Brackett, as mentioned by Dr. Connelly. Although such procedures work adequately in *in vitro* systems to minimize effects of polarization of the platinum cathode, they have been quite unsatisfactory in our hands when the oxygen electrode is in tissue. In the latter situation, large uncontrollable deflections occur in the galvanometer at each switching phase. We now merely close and leave closed the circuit, allowing the initial polarization to proceed and level off after 15–40 minutes; from then on little drift is usually seen and the system performs satisfactorily. Another practical point which has not been mentioned is that in any given placement of the electrode below the tissue surface, a blood vessel may be ruptured. This leads to accumulation of a pool of blood around the electrode tip; the resulting diffusion barrier leads to varying degrees of loss of sensitivity of the electrode. The presence of cellular detritus may have the same effect. When such a situation appears to exist, the only recourse is to remove the electrode, clean it and replace it in a different area.

DR. CONNELLY: The oxygen electrode enclosed with its reference electrode behind a polyethylene film may not perform ideally in every conceivable application. Polyethylene is permeable to water vapor to some extent and in a dry gas phase or in solutions not isosmotic with the electrolyte layer under the film, the volume of this thin layer may gradually change, thereby changing the diffusion geometry and the sensitivity. Substances such as NH_3 or HCN may well traverse the polyethylene barrier as vapors and effect the reaction at the platinum or at the silver. A further question of importance is, what is the effect of the decrease in concentration of chloride ion in the electrolyte layer, as chloride deposits as silver chloride and is replaced by hydroxide? In one test potassium hydroxide was used beneath the polyethylene film, instead of sodium chloride. The current-voltage curve showed little indication of a plateau, but at −0.8 v. polarization the electrode appeared to respond properly during cycling between air and nitrogen. The question deserves further study.

DR. SHEPARD: The factors which seem most likely to produce inaccuracy in estimating tissue oxygen tension are: *1*) Inaccuracy of electrode calibration, including all of the sources of error mentioned previously. One must also consider the effect of variations in temperature of the tissue, since most oxygen cathodes have quite a high temperature coefficient of calibration; *2*) alteration of tissue Po_2 because of the trauma produced by insertion of the electrode and any inflammatory reaction produced by its presence; *3*) variations of Po_2 in different parts of the tissue at different times. The electrode may not be located properly to average spatial variations accurately and its response characteristics may interfere with accurate averaging of temporal variations in Po_2.

AMERICAN PHYSIOLOGICAL SOCIETY

Symposium on Utilization of Recorded Knowledge[1]

Chairman: MAURICE B. VISSCHER

INTRODUCTION

MAURICE B. VISSCHER

From the Department of Physiology, University of Minnesota Medical School, Minneapolis, Minnesota

THIS symposium has its justification in the fact that there is a growing degree of non-utilization of recorded knowledge. The time is long past when any scholar can hope to read, let alone comprehend and assimilate intellectually, all the literature in his own and related disciplines. At the present time, whether we like it or not, and whether we realize it or not, we are all of us living on an intellectual diet which is restricted and selected, not only by our own choices, but by the force of circumstances beyond our personal control.

As science has grown into an enterprise occupying the time and efforts of millions of men and women around the world, the problem of utilizing the enormous mass of new information has become more and more complex and even frustrating.

The practical problem of making knowledge available to its potential users has many facets. First of all there are many different kinds of users and uses. Each kind must somehow be served. The beginning student, the teacher, the specialist investigator, the philosophic integrator, the professional practitioner, the industrial manufacturer and the popular consumer, each and every one has somewhat unique needs.

This symposium is a recognition that the problem is realized by scientists as one which is important for scientists themselves to consider. It is also a small beginning toward a resolution of the problem.

There have been brought together here today several experts in the field of documentation and information theory. We hope to have some enlightenment on the uses and inadequacies of our present bibliographic control mechanisms and some clarification and definition of the problems that await solution.

[1] Chicago, Illinois, April 18, 1957.

SYMBOLIC SHORTHAND SYSTEM FOR MEDICINE AND PHYSIOLOGY

HANS SELYE AND R. K. MISHRA

From the University of Montreal, Montreal, Canada

ONE of the most important and urgent tasks of contemporary medicine is to create an index of its contents. If the number of medical publications continues to increase at its present rate, it will soon become impossible for anyone to keep up with current progress, even in the most limited field. Yet the full exploitation of discoveries and the logical design of new experiments must develop upon the knowledge of available facts. The time has come to face this situation as a major scientific problem characteristic of our century; otherwise, "science may become bogged down in its own product, inhibited like a colony of bacteria by its own exudations" (1).

Virtually every physician and investigator has some sort of personal index in which he can file away interesting publications for future reference. In most cases, these systems consist of abstract cards and reprints (roughly arranged according to major subjects), and a cross index of the authors. The author index of such files is almost always satisfactory, but the divisions for the subjects are generally quite vague, so that the owner must rely largely on his memory to find any one paper. These personal indexes are not usually constructed on the basis of any set of written rules, and the systems of classification tend to change from time to time with shifts in the owners' interests. Very few private files that we have seen were considered to be adequate even by the most inexacting owners.

In order to prepare for a rich harvest of really significant findings, we must constantly consolidate our gains by cataloguing them effectively. A great deal of creative thinking by many original minds will be required for the development of a truly satisfactory system, which is fully adapted to the needs of all branches of medicine. The bibliographer who systematizes knowledge so that it may serve as a basis for the design of experiments, the physiologist who devises animal experiments to prove that a given body constituent exerts certain desirable actions, the chemist who synthetizes this constituent, and the clinical investigator who finds out how to use it for treatment, are all doing research which can be equally original and equally useful to medicine, though the methods they use are quite dissimilar.

To our mind, the reason why we still do not have an adequate system for orientation in the medical literature is that the superficial kind of indexing by main subjects or titles is grossly inadequate and a more detailed cataloguing of available information is impossible without the use of a simple international code. Added to this is the singular difficulty of obtaining financial support for the organization and dissemination of knowledge. Most foundations and governmental institutions, who distribute many millions of dollars every year to subsidize medical research, are quite unwilling to spend anything to make the knowledge gained by research generally accessible. It is our impression—or at least, our hope—that this attitude might change with the development of a cataloguing system which could furnish the required key to available information if it were adequately subsidized.

The Symbolic Shorthand System (SSS). The SSS is not meant only for large-scale library work. It is a system applicable to any problem of medical documentation.

Instead of the usual code designation composed of numbers, letters and other signs which in themselves have no meaning, the SSS uses mnemonic symbols and signs reminiscent of the subjects they denote. For example: Adr = Adrenal, Tr = Thyroid, ↑ = increase, Tr↑ = hyperthyroidism.

All possible subjects and relationships between subjects are first arranged in one dimension, according to a rigidly fixed order of precedence so that they may be typed out on one line of a card (the subject card). For example: the effect of thyroxin (TX) upon the adrenals of the rat would be written: Adr ← TX /Rat.

The statements are then arranged by filing the subject cards one after the other according to the fixed order of precedence.

Since all symbols have only one fixed meaning they eliminate the possibility of synonymous designations. In coining such symbols we have been guided by the following principles:

1) Elimination of language barriers. These symbols transcend language barriers because they are abbreviations derived from internationally understandable Greco-Latin roots and technical terms (Cr = cardiac, R = renal, Ach = acetylcholine).

2) The most frequently used symbols must be the shortest.

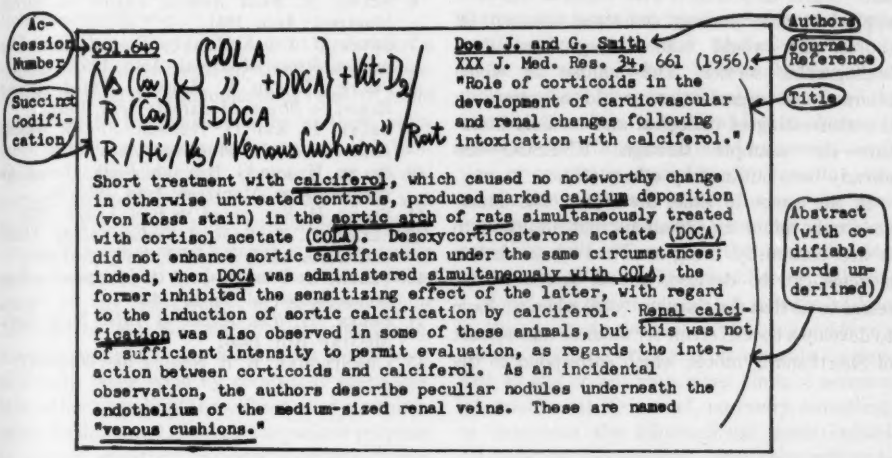
3) Use of signs to promote brevity and clarity, e.g., ↑ = increase, A ← B = action of B on A, A > B = B content in A.

4) Selection of phonic letters and syllables of mnemonic value for the coining of symbols.

Any theoretical interpretation is subject to revision. Hence, if we were to list observations as being in agreement or disagreement with a theory, they would lose the very basis for their classification if this theory were later disproven. To avoid this we always classify the paper according to the observations made by the author. Although facts are the basis of our classification, we must recognize that most observations are devoid of any fundamental meaning unless they are connected by a theory or hypothesis. Hence, significant interpretations are also indexed.

A preliminary report of this method of classification has been published (2) and a fuller description of the method may be obtained on request from this institute, so it is not necessary to go into details here. The following will serve as an outline of the method of codification and filing according to the SSS.

Our library consists primarily of original texts. All publications receive consecutive Accession numbers as they arrive in the Library and are filed on shelves in order of these numbers. The information in the documents is transcribed into the SSS code by codifiers. The following is a typical example:


Accession Number
C91,649
Succinct Codification
Vs (Ca) / R (Ca) ← COLA / " +DOCA / DOCA + Vit-D2 / Rat
R/Hi/Vs/"Venous Cushions"
Doe, J. and G. Smith
XXX J. Med. Res. 34, 661 (1956).
"Role of corticoids in the development of cardiovascular and renal changes following intoxication with calciferol."
Short treatment with calciferol, which caused no noteworthy change in otherwise untreated controls, produced marked calcium deposition (von Kossa stain) in the aortic arch of rats simultaneously treated with cortisol acetate (COLA). Desoxycorticosterone acetate (DOCA) did not enhance aortic calcification under the same circumstances; indeed, when DOCA was administered simultaneously with COLA, the former inhibited the sensitizing effect of the latter, with regard to the induction of aortic calcification by calciferol. Renal calcification was also observed in some of these animals, but this was not of sufficient intensity to permit evaluation, as regards the interaction between corticoids and calciferol. As an incidental observation, the authors describe peculiar nodules underneath the endothelium of the medium-sized renal veins. These are named "venous cushions."
Authors
Journal Reference
Title
Abstract (with codifiable words underlined)

Now the card is turned over to a filing clerk, who prepares seven subject cards—one for each unit of information contained in the succinct codification. The following is an example of one of the seven cards:

Vs (*Ca*) ← COLA + Vit-D2 /Rat

Doe, J. and G. Smith C91,649:56.

The filing according to the order of precedence will put each card into a division of the subject file where it can easily be located, together with reference to any cognate observations. An investigator interested in any one of the seven subject units selected for registration will have to consult the file at one point only, because the rigid order of precedence obviates the possibility of filing an item anywhere except in its one determined position.

The basic pattern of the SSS has proved its worth in the last 25 years in the cataloguing of some 350,000 original articles and in helping members of our institute to retrieve information

from them. Let it be mentioned here, incidentally, that the system was indispensable for the compilation of several works in which a total of well over 50,000 references have been individually classified (3/11) and in writing books which incorporate (without individual references to each paper) the salient facts selected from among the 350,000 entries in our library (12/14).

We see no reason why the SSS could not be applied to all other fields of medicine with the same degree of precision with which it has been applied to endocrinology and stress research. Of course, this would take a large centralized bibliographic service. The manner in which international organizations could cooperate in the abstracting of biological and medical literature—for example through UNESCO—has already been outlined by others (15).

In the course of time, the system has undergone only minor modifications, but additions to it are constantly being made. Perhaps better systems can be devised, but in any event, it seems to us that the time has come when we have to develop a special script for medical topics, a set of Shorthand Symbols, which correspond to the structure formulae and equations of chemistry, the symbols of algebra and the notes of music.

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FLOW OF INFORMATION ON CURRENT DEVELOPMENTS IN THREE SCIENTIFIC DISCIPLINES[1]

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The pilot study on the flow of information in certain scientific disciplines in which we are currently engaged had its origin last year. At that time, representatives of the National Science Foundation became interested in some of the work on communications being carried on at the Bureau of Applied Social Research of Columbia University by means of interview surveys. This work has focused on tracing the diffusion of messages and on studying the effects of various means of communication. Work has been done among large publics of consumers and citizens and, more recently, among specialized groups. One recent example was a study of the spread of information regarding new drugs among physicians. The doctors' problem in keeping up with developments in medicine is a very acute and keenly felt one. The scientific professions which the National Science Foundation is designed to serve, it was thought, are also beset by problems in the flow of information. To be sure, the information-gathering problems of research scientists differ in many ways from those of practicing physicians. It nevertheless appears that the ever-increasing abundance of information which is available is making it more difficult for the individual scientist to be as well informed as he would wish. The rate of scientific output has made it more difficult than ever for scientists to keep abreast of new developments in their own special-

[1] Publication no. A 232 of the Bureau of Applied Social Research, Columbia University. The work reported is being done under a grant from the National Science Foundation.

ties, to be aware of additions to knowledge in neighboring fields, and to maintain contact with the broad trends in their disciplines. The problem is clearly not that information isn't made available—somewhere. It resides rather in the physical impossibility of any one man's keeping up with more than a fraction of the output, and in the consequent necessity of selecting and screening what is available, of becoming aware of what there is to select from, and of finding and obtaining information in the most efficient and least time-consuming way.

It was accordingly decided to undertake a pilot study to see what, if anything, the interview survey method of communications research could contribute to the understanding of these problems. Our data come from interviews with biologists, biochemists and chemists at several institutions in the New York area. Work was begun on February 1; today's report is essentially confined to announcing what it is we are trying to do. It should be made clear that this is not a study of the technology of communications facilities; on the technical problems of storing, indexing and processing information much work is already being done by competent specialists. It is rather a study of the *utilization* of communication facilities and channels for various purposes by various scientists. Included is an exploration of the role of informal, person-to-person, and unplanned forms of communication as they articulate in the total communications process with the more standardized channels such as journals, conferences, etc. These topics not only hold the greatest inherent interest to us as sociologists in the furtherance of our own scientific goals; it is also hoped that we can here serve the sciences best by drawing for ideas and hypotheses on the work that has already been done on communications processes among non-scientific publics. Here a series of studies has specified in some detail the ways in which informal and formal means of communication interlock to produce given effects.[2]

This also points to a fundamental difference between the present task and the preceding research referred to. For in the previous instances the research objective was, reduced to its simplest terms, to determine what sort of communications were necessary to bring about a specified effect; while for the communications among scientists, it cannot be stated in advance what effect is to be attained, except in very abstract terms. The earlier studies—to mention some examples—investigated the channels of communication through which voters are led to change their vote intentions; they inquired into the sources of influence which determined the motion picture shows a population of teenagers attended during a certain month; they inquired into the sources of information which, alone or in varying combinations, persuaded medical doctors to adopt, or not to adopt, a new pharmaceutical product. Now of course the decisions faced by scientists in their research differ in dozens of essential ways from those faced by voters, movie fans, or practicing physicians; but from the communications research point of view, the most important difference is that a number of research scientists will seldom face the same decision, since they will be working on different problems.

Hence it is unfortunately not possible, at least at this point, to determine what information is 'needed' by comparing the performance of those who do and do not have given kinds of access to information. It is, instead, necessary tentatively to determine the informational needs individual scientists are aware of; only then will it be possible to see in what ways information can be classified and categorized so that at least needs for certain *categories* of information can be said to be held in common by whole classes of scientists. Given some realistic classification of the types of information that scientists engaged in varying kinds of work need, it then becomes possible to attack a further question: how adequately do varying channels of communication fulfill each of the informational needs for scientists in each of a variety of situations?

One of the tasks of the pilot study is, therefore, to determine how scientific 'messages' are to be classified so that it becomes meaningful to say, "This medium of communication is useful in satisfying the needs of such and such scientists for messages of this kind but not for messages of that kind." Preliminary consultations showed that messages must, for this purpose, be differentiated along at least three important dimensions:

1) The distance from the scientist's own field—i.e., whether the message refers to the scientist's own specialty, to neighboring fields, or to developments in more remote areas of science. *2) The*

[2] Much of this work is reviewed in *Personal Influence: The Part Played by People in the Flow of Mass Communication*, by Elihu Katz and Paul F. Lazarsfeld. Glencoe, Ill.: Free Press, 1955.

content of the communication—i.e., whether it refers to experimental results, to procedures, to theoretical reformulations, etc. 3) *The function* the message can serve—i.e., whether it conveys actual scientific knowledge, arouses interest in it, tells where to find it, calls attention to its existence, etc. Empirical questions that arise in this connection are, for example: How broad a field is to be considered a man's 'specialty'—i.e., just what would be included and what excluded from the range of work within which a scientist feels responsible for keeping up in great detail? And what is the range of a wider field within which he feels at least responsible for knowing what is going on? Do messages about findings, procedures, and theory wane equally in importance as they move from a scientist's specialty out to more and more distant fields?

Our survey uses several different approaches to answer questions like these, and to find out what other differentiations of information may have practical importance, in the sense that they are relevant to the usefulness of various means of communication. One of the interviewing techniques consists of presenting the scientist with a list of specialities in his discipline; he checks off those where he tries to keep up with current developments in detail. Then he marks other specialties that he keeps up with, though in less detail. When he has done so, he is asked to characterize the difference of his informational requirements in the two or more sets of fields. There are many different ways in which such a differentiation might be made; some of them are illustrated by the following comments made by scientists interviewed to date: "I follow things in microbiology only insofar as they help in my own field (cytochemistry). Chemical aspects of cells have been well worked out in microbiology, but I am not interested in microbiology as such. But what I do want from it I want to know just as promptly and accurately as anything else." Or, "I am not interested in the field of . . . as such, but only follow things in it if they deal with the organism I work with." Contrast this with differentiations according to the amount of critical scrutiny that the scientist is able to give to a given field; e.g.:

> In my primary field, I would certainly bring a degree of critical attention, would judge everything critically. In these cognate fields I want to know where they are going and how fast they are getting there, and get a much more general picture. I do need to know how they are progressing, because many of the problems are joint problems to all these fields. Of necessity, I have to take their findings less critically, although I will want to know that an expert who has read it finds the procedures ok. It is in these fields, therefore, that critical reviews are of special importance to me. Not mere compilations.

A similar differentiation is often made by the scientists interviewed when they assert that, in the secondary fields, they are "interested only in the results, not in how they did it." At the same time it must be realized that in some instances it is precisely "how they did it" that is significant for a person in another field. Thus a man working in physiological genetics said:

> In embryology I have to be aware of developments and it is for technical reasons; I need the data themselves, in order to use them as diagnostic criteria of the way in which my genes act, which I have to diagnose in embryos. In population genetics, on the other hand, I need to keep up with theoretical developments and not so much with data. That gives the pattern within which my own work will be interpreted.

Still another way of selecting what is significant to keep up with in a secondary field is expressed as follows: "In this field I want to know what the major figures are doing; I don't have to know what everyone is doing." As our study progresses, additional differentiations of this sort will, no doubt, be encountered and, it is hoped, some picture of their prevalence among scientists in different situations will be obtained. It may, e.g., be that those who teach differentiate in one way, those who do only research, in another. It is even more important to learn how the usefulness of various channels of information relates to such differentiations. Thus, for example, it may well turn out that when a specialty is of secondary interest to a scientist because of its possible theoretical bearing on his own field, he is most effectively kept abreast of it by following annual review volumes or transactions of symposia; while when a specialty is of secondary interest to a scientist as a diagnostic tool he will want to know precisely the technical details that reviews might omit, so that he will—if he is not able to follow the original literature—do best to keep in personal touch with some important men in that specialty, perhaps by periodic visits to their laboratories. In this connection it is important to realize that the very same specialty—blood chem-

istry, for example—may be the field of primary interest to one set of biochemists; a field of secondary interest because of its theoretical implications to a second set of biochemists; and a field of secondary interest as a diagnostic tool to a set of geneticists. This raises the interesting question whether work in any one specialty ought not, perhaps, to be reported in more than one format, packaged, so to speak, for different sets of consumers. But it is too early to speculate further along this line.

One other important dimension along which scientific 'messages' and the need for scientific communication must be differentiated is, as stated earlier, the functions which the message can serve or the uses to which it is put. Under this heading falls the well-known distinction between reading to keep abreast of current developments, and reading for 'reference,' i.e., to find any available answers to a specific question. But scientific communication must serve the scientist in other ways as well: e.g., by advising him what work is being carried on, where, and by whom; by informing him of the identity and location of experts in various lines of work, whom he may wish to consult when the need arises; by directing his interest, from time to time, to new topics which he had not previously considered to be in his sphere of interest. Such distinctions appear important to an understanding of scientific communication, since communication research in non-scientific areas has repeatedly shown that different communications functions are performed, at their best, by different channels and media of communication. Our pilot study is therefore putting considerable effort into devising ways to determine the different functions which communication serves for research scientists, and the communication channels through which each function is typically performed. This leads, for example, to problems like the following: What is the role of diverse sources of information in arousing new interests? Are general-science journals like *Science* or *Scientific American* of any importance here? Or is it personal interchange that plays the leading role? And if the latter, what contexts are most conducive to conversations which will arouse new interests? Are there any systematic ways of calling the scientist's attention to new approaches of which he had not been aware?

In the course of the study there have already emerged some functions of scientific communication which we had not anticipated. Thus it seems, for example, that personal encounters during a scientific meeting, even when they are not productive in terms of information exchange on the spot, may facilitate later communication by correspondence. Thus one man said:

> When I have talked to someone at a meeting, I get an idea of how sophisticated he is and how much he knows about the kind of work I do, and when he writes me later with a question I . . . know just at what level to pitch the answer. If someone writes me cold for some explanation, it's a hell of a chore to answer him.

Here we see how one means of communication —conversation at a meeting—serves to facilitate another means of communication—correspondence. This little remark thus exemplifies the scores of ways in which different means of communication interlock. This is perhaps what gives person-to-person communication among scientists the great importance which seems to be quite widely acknowledged by scientists. One biologist went so far as to declare: "I don't consider one a specialist in a given area unless he also knows a good many other specialists in it." Person-to-person communication appears to play a considerable role even in scientists' use of the more formal means of communication, including, of course, the professional literature. Thus many a journal article is read because it was mentioned in a conversation; a report on work somewhat removed from the reader's specialty is often not fully credited until a trusted acquaintance pronounces it reliable; and a novel procedure that is mentioned in a report on an experiment may send the reader scurrying to the author's laboratory to find out its technical details. If it is true that person-to-person communication is important even in the consumption of the knowledge which is recorded in black-and-white, then it is also important to know what are the main occasions for person-to-person communication, and what factors provide some scientists with ample opportunities for such interchanges, while leaving others in a relatively isolated position.

In attempting to develop some knowledge on this point, it is possible to draw on the sociology of communications for some guiding ideas. One of these is the realization that each person plays not one, but a number of roles, vis-a-vis different sets of people, and that these provide so many potential channels of communication. It is therefore worthwhile to subject the activities of scientists to a sort of job analysis in terms of the

occasions for personal interchanges with other scientists which they provide. Teaching, as well as research duties, must be examined from this point of view. In addition, many scientists engage in other, usually more tangential, activities; some of these provide them with access to information, sometimes in unexpected ways. Thus, for example, some of our interviewees are members of committees which screen applications for grants. Ostensibly, this is an administrative chore, but sometimes meetings of such a committee provide its members—especially if they come from different parts of the country—with a singular opportunity to chew over each other's research work and to pass on news of the work of others. One such committee member reports:

> These committee meetings are absolutely priceless to me for information exchange. We educate ourselves there despite the original intent. It is so rewarding that we have decided to extend one of our meetings each year into a two-day private symposium.

A member of the editorial board of a journal comments similarly:

> In my capacity as editor I have lots of correspondence with the referees. That way I learn their opinions, and frequently learn new methods from the referees who apply them in their judgments of the papers submitted to them.

Chairmen of program committees and organizers of conferences have similar opportunities, especially if the conference or section they deal with has a reasonably specialized focus and has some continuity over the years. One such person commented:

> I am in a good position with respect to this work because I am the organizer of three past conferences and we are now planning a fourth. And the people feed me news about work related to this problem. They keep me informed by asking questions. We usually send out a form, "Should a new conference be held? Do you know any other workers that should be invited?"

Such organizers not only have added opportunities to learn of the work and views of many in their field, but they can also pass such information on to others and become focal points of scientific communication.

Opportunities for personal interchanges between scientists are also affected by spatial arrangements and time schedules. Thus, for example, at a school at which the various offices and laboratories of the biological disciplines are dispersed over a large number of buildings some distance from one another, there colleagues will not often have casual encounters. At one such school one of the younger men said:

> It is not my colleagues here who call things to my attention. They would already have been called to my attention before I will speak with a colleague about them. They are very accessible when you have a specific thing to bring up; but there are very few occasions for incidental shop talk. At *X* University, all are in the same building and have tea together, so it is easy to exchange talk. If you want to talk with somebody here, you have to make a special effort, call him up, have a purpose in mind.

A third set of factors that affect a scientist's opportunities for exchanges of information with other scientists is the internal social structure of his own department or division. One of the conditions that enters here is the question of what sociologists call status differentials. This may be illustrated by a remark made by a scientist who, as a younger man, had worked in one of the top-ranking departments: "Some of the men there," he reported, "were extremely famous and always busy giving talks or something. The rest of us were kind of isolated from talking with them." This may not be the common experience of young men in top-ranking departments, but it does seem worth while to inquire whether smooth communications within a department are enhanced or hindered by the presence of men at a middle rank between juniors and seniors.

Differences in the customs of information-exchange that prevail between different disciplines must also be considered. Thus, for example, biologists have traditionally sent reprints of their publications to a more or less permanent mailing list, while chemists, for the most part, send reprints only on request; and, of course, do not expect to get reprints from others unless they specifically request them. These are strictly matters of custom. They do, however, become problems for people who bridge the fields; for example, a biologist who in recent years has become most interested in chemical aspects has to keep tabs on which authors he has to send request cards to, and which authors he can rely on to send him reprints automatically. Similarly, people have to adapt their reading habits when,

for example, they have been used to reading in a field where it is traditional to publish only fairly comprehensive results and they now broaden their interest to include a specialty where it is the custom often to publish individual findings.

SUMMARY

These few examples may illustrate some of the aspects of the communications behavior of scientists which are being investigated: *1*) the scientist's need for information of various sorts about developments in his own and other specialties; *2*) the suitability of various means of communication—printed and oral, official and personal—to satisfy each of these needs; *3*) the interplay between the formal and informal ways of communicating; and *4*) the situational factors which affect a scientist's access to means of communications of various sorts. These are among the chief topics being explored. The present pilot study is being carried out in order to develop a more thorough listing and objective definition of the variables to be considered, a systematic set of hypotheses about their interconnections, and appropriate procedures for an eventual large-scale survey.

GROWTH OF BIOLOGICAL LITERATURE AND THE FUTURE OF BIOLOGICAL ABSTRACTS

G. Miles Conrad

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Although the present paper is concerned with the future growth of *Biological Abstracts*, it is useful for us to review the history of biological publication from its beginnings in order to guess at its possible development in the future. Skipping over the clay tablets of Assyria and the herbals and bestiaries of the Middle Ages, the early communication of biological information was conducted by direct oral discussion and by personal correspondence. However, as more and more scholars became interested in the natural sciences this process of personal communication did not suffice and the formation of philosophical societies provided forums where the ideas and findings of individual workers could be readily conveyed to group assemblages of their colleagues. Soon these societies for purposes of record, began to publish minutes of their transactions periodically, and these published proceedings with their contained scientific knowledge became a most convenient substitute for personal correspondence—indeed, they were a species of circular letter.

It is at this point that we can begin to have a numerical idea of the amount of communication conducted by scientists in general and biologists in particular. During the eighteenth century these periodical transactions remained few in number and general in content, but soon their great value was proven as a relatively rapid communication medium for the dissemination of new ideas and new discoveries. Thereafter we see, during the nineteenth century, a steady increase in their numbers and a growing tendency away from the journals of general science toward journals specialized as to subject coverage.

Figure 1 illustrates graphically the growth of periodical scientific and technical journals published in the United States from 1800 to the present and shows a considerable similarity in the several growth curves throughout the 150 years represented. Of course, it must be remembered that this growth is not due merely to the advantages of the journal as a communication medium, but must also be due to the pressure for publication from an increasing body of research workers, inventors and natural philosophers. Its growth must also have been fostered by improvements in the technologies of printing and paper-making and the resultant reduction in the relative cost of printing.

The next chart (fig. 2) brings us closer to our interests, for it represents the growth of the world's biological research journals, exclusive of medicine and agriculture, over a period of nearly 200 years. Probably the most notable attribute of this curve is the fact that the number of journals published has doubled on an average of

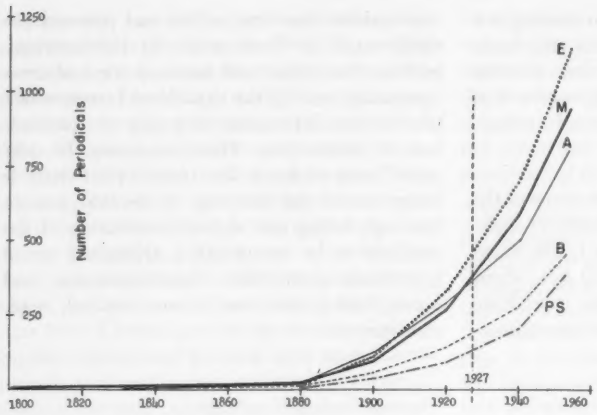


FIG. 1. Growth of scientific and technical periodicals in the United States from 1800 to 1954. Data from (5). *Key: E*, engineering and technology; *M*, medicine; *A*, agriculture; *B*, biology; *PS*, physical sciences.

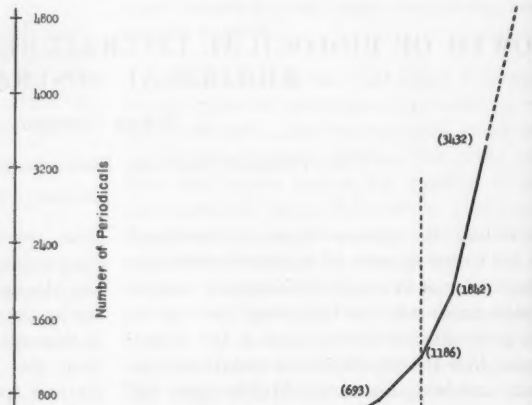


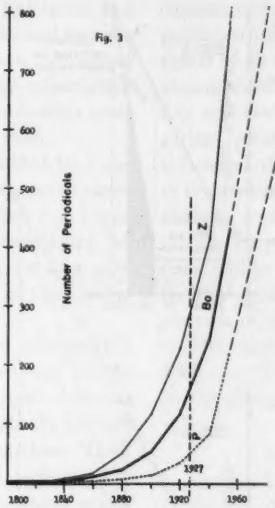
FIG. 2. Growth of the world's biological research periodicals (exclusive of agriculture and medicine) from 1760 to 1954. Data from (6).

every 18 years. From the point of view of *Biological Abstracts*, however, the most significant thing about this curve is the fact that only 35% of the biological journals published today were being published in 1927, the year of its establishment. I must emphasize that 65% of the biological research journals published today have come into being during the 30 years that *Biological Abstracts* has been functioning. This is the crux of our problem and I will return to it in a moment.

Because I have promised to refer to *B.A.*'s future coverage of physiological literature, the next chart (fig. 3) may be of special interest, for it is a breakdown of some of the data recorded on figure 2 into several specific areas of biology—zoology, botany and animal physiology. It is interesting to note that although the actual numbers of journals vary, the profiles of the three curves are remarkably similar. As you know, physiology has shown an especially phenomenal growth during the 30 years since 1927. Although the absolute number of journals currently published in this field is only 219, this figure is more than five times the number published in 1927.

During this same period *Biological Abstracts'* relative coverage of physiological literature has grown from 1927, when it accounted for 19% of the contents, to 1956, when it accounted for 26%. Inasmuch as the increase of physiological journals constitutes about 8% of the total increase of all biological journals during the past 30 years, it is apparent that physiology has received its fair share of a proportionate increase in coverage, and this will continue to be the case.

Recorded information that is reasonably easy of access has enabled us to learn something of the



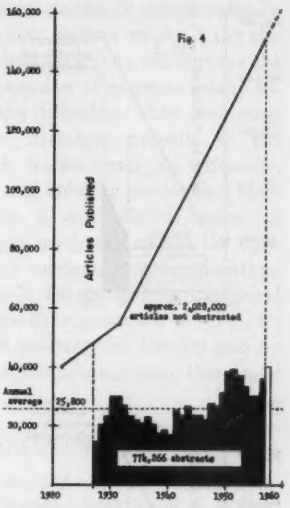


FIG. 3. Growth of the world's specialized biological research periodicals from 1800 to 1954. Data from (6). *Key: Z*, zoology (exclusive of physiology); *Bo*, botany; *P*, physiology (animal).

FIG. 4. Estimated growth of biological research literature from 1921 to 1956, and its coverage by *Biological Abstracts* from 1927 through 1956.

growth of journal publication. However, the unit of information which must perforce concern the abstracting and indexing service is not the journal but the individual journal article. Here the information is less well developed. From the few estimates assembled (2), figure 4 has been prepared to show the possible volume of research articles published in pure and applied biology during the period of *Biological Abstracts*' activities as compared with the actual coverage of this literature by *B.A.* Although *B.A.*'s record of abstract coverage exceeds that of any other service in biology, it has been able to cover only about 26% of the total volume of this literature.

Schramm (3) estimated that approximately 55,000 articles of biological research interest were published in 1930. Zwemer (7) has estimated that in 1954 there were about 144,000 such articles. Although these estimates were arrived at independently of each other and without direct reference to the journal growth figures discussed above, it is most interesting to note that Schramm's estimate of articles published for 1930 is 38% of Zwemer's estimate for 1954. The journal survey shows that the total number of biological journals published in 1930 is 38% of those published in 1954. So close a similarity in percentages between article and journal-growth is probably coincidence, but in the absence of conflicting data it can be assumed that the abstracting task of *B.A.* has almost tripled in the 30 years of its existence.

Unfortunately, during most of these same thirty years *Biological Abstracts* was fighting for its financial existence. As a result, although averaging 26,000 abstracts per year, *B.A.*'s relative coverage of the estimated total output of research writings dropped alarmingly from about 45% in 1930 to about 17% in 1954. In 1957, with the publication of 40,000 abstracts, *B.A.* will cover approximately 25% of the current research literature.

What of the future? Figure 5 projects the growth of biological research article publication into the first decade of the twenty-first century. If the growth of biological literature continues at the same rate it has assumed in the past 30 years we can expect that in the year 2010 alone about 348,000 articles will be published. Perhaps before that time some new and more efficient method of communicating information will have come into being—possibly ESP? In the meantime, though, for the next 30 or 40 years we are probably tied to the book format for the widespread communication of biological intelligence. What can *Biological Abstracts* do to keep abreast of this staggering task of abstract coverage?

In order to plan the orderly growth and development of *Biological Abstracts* as it begins its fourth decade of service, the staff and trustees have recently completed a careful study of past experience with reference to income sources, personnel requirements, production costs, etc. The data derived from this study have been applied to the estimated future growth of biological research literature in order to ascertain

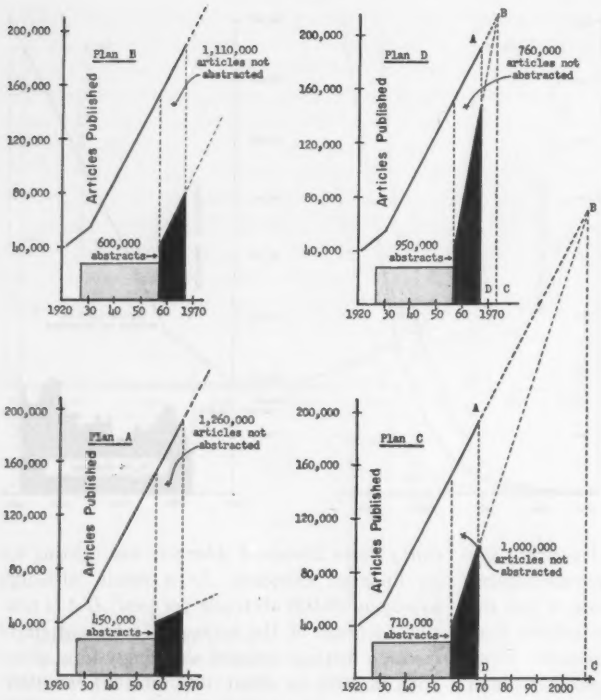


FIG. 5. The possible growth of biological literature from 1957 through 1966 and its coverage by *Biological Abstracts* under *Plans A*, *B*, *C* and *D*.

the optimum goal in coverage that *Biological Abstracts* could reasonably hope to reach during the next 10 years. Figure 5 has been extracted from this study and illustrates four of the possible growth rates for *Biological Abstracts* that were considered.

Plan A—Abstract coverage would increase at approximately *B.A.*'s present rate, rising from 40,000 in 1957 to 50,000 per annum by 1966. During this 10-year period, *B.A.* would publish 450,000 abstracts or 26.3% of the 1,710,000 biological research articles that probably will be published during the next decade. The rate of *B.A.*'s growth would be so slow that it would never catch up with the literature's growth.

Plan B—Abstract coverage would increase from 40,000 to 80,000 during the next decade, and *B.A.* would publish 600,000 abstracts, or 35.1% of the total. The rates of growth of both the total literature and *B.A.*'s coverage would be essentially parallel and would never coincide.

Plan C—Abstract coverage would increase from 40,000 to 100,000 during the next 10 years, and B.A. would publish 710,000 abstracts, or 41.5% of the total. The rate of growth of *B.A.*'s coverage under this plan would coincide with that of the total literature in the year 2009 A.D. (As an interesting statistic, it should be noted that the polygon, ABCD, represents approximately 11,653,000 research articles in biology which might be published during the 43 years from 1967 to 2009. Of this huge volume of literature, *B.A.* would be required to cover 9,675,000 articles, or 83% of the total. At $6.50 per abstract the cost of this program would be $62,887,500 or an average of $1,462,500 per year.)

Plan D—Abstract coverage would increase from 40,000 to 150,000 per year during the next 10 years, and *B.A.* would publish 950,000 abstracts, or 55.5% of the total. If this plan were adopted, however, *B.A.*'s rate of coverage growth would coincide with the total literature's rate of growth in 1973. (In this instance, the polygon, ABCD, would represent about 1,400,000 articles, of which 1,267,000, or 90.5% would be covered by *B.A.*)

After careful consideration of these four plans, the Trustees have adopted *Plan B*—an increase from 40,000 to 80,000 abstracts per annum—as a working goal for *Biological Abstracts* during the next decade.

With the volume of biological writings increas-

ing at an exponential rate on the one hand, and the costs for personnel, commodities and services increasing sharply on the other, *Biological Abstracts* must either increase its subscription income substantially or cut its production costs materially. We are striving to do both.

Subscription income can be increased by a rise in subscription rates and/or an increase in circulation. Rates were raised in 1956 and it is hoped that the present level can be maintained for several years more. Campaigning for new subscribers is a never-ending activity of the Central Office staff.

Production costs can be reduced substantially by increasing the relative productivity of the Central Office personnel. *Biological Abstracts* envisions such an increase in productivity through an increasing application of automation. Thus, by the adoption this year of IBM techniques in the production of the monthly and annual author indexes a saving of 254 typist-days is anticipated. This saving is being applied to increased abstract and subject index production. Similar applications of automation will be made to other activities as soon as feasible and practical programs have been developed. The use of author's abstracts, too, although spurned by some abstract services, has been an important factor in helping *Biological Abstracts* to keep costs down during the past. Increased use of author's abstracts can be counted upon to further reduce costs in the future.

Although perhaps not completely pertinent to the topic of my paper it should be noted, in conclusion, that the very success of the periodically published journal may contain within it the elements of its own destruction. As one surveys the almost incredible number of journals extant today and realizes the difficulties which this staggering volume of literature presents to the individual research worker—and, by extension, to the abstracting and indexing service that must analyze and make it available—it seems as though we will soon reach and exceed the optimum utility of this medium of communication. In 1952 there were 50,000 scientific and technical journals in existence throughout the world (1, 4); in 1970 there will probably be 100,000 and by 2006 A.D., if the doubling continues, there may be 400,000.

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PROBLEMS IN COMMUNICATING RUSSIAN SCIENCE

SCOTT ADAMS

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IN ITS report on the 1957 appropriations bill for the Department of Health, Education, and Welfare, the Senate Appropriations Committee remarked that "failure to promptly make available to American scientists the full findings of Russian scientists opens the door to useless duplication of work, waste of time and money, and delay in grappling effectively with the major disease problems of today." It directed the National Institutes of Health "to provide, through grant or contract, a Russian medical research translation service" (1).

The immediate antecedent of this legislative action was testimony from American medical scientists who had recently participated in an exchange mission (2) that the Russians had perfected mechanisms for reporting in detail the "full findings" of American research scientists, while the American scientific community was generally uninformed concerning the accomplishments of Russian scientists. Earlier antecedents were the Atoms for Peace Scientific Conference at Geneva in August 1955, where American atomic scientists learned of the accomplishments of their Russian counterparts, and the successful Russian translation programs established as an outgrowth of this Conference by the Atomic Energy Commission and the National Science Foundation (3). A further development of significance in abetting the exchange of scientific information between the United States and the Soviet Union was the Presidential approval of a National Security Council recommendation calling for the free exchange of information, of books, journals, government publications, exhibits, and of persons in professional, cultural scientific and technical fields (4). Despite the tensions occasioned by the Hungarian revolution, this unilateral application of the Geneva 17-point program has met with comparable pronouncements on the part of the Soviets, and has facilitated the exchange of scientific publications and persons necessary for the success of a translation program.

In July 1956, therefore, the National Institutes of Health received a mandate to establish a translation program for the purpose of making available to American medical scientists the full findings of Russian scientists. This is an account of some of the problems met in developing this program.

Essentially the task is one of translating the information carried in one national scientific communication system (5) to another. This implies a broad-based approach, since there are both a wide variety of information to be communicated and a number of different techniques available for its communication. The NIH program, detailed at the end of this paper, has included the cover-to-cover translation of eight research journals, the provision of an abstract service, the publication of a dictionary, a directory, and the support of review papers. Despite the variety of these activities, the problems involved in their planning and administration are general, and fall into the following four categories: selection, form, distribution and acceptability.

Problems of Selection. Russian medical literature is voluminous. An estimate based on a count of the titles in *Knizhnaya Letopis*, the weekly record of Russian book production, shows an average of 2,300 titles per year classifying broadly in medicine. The export subscription list *Periodica* lists a selection of 83 titles in the bio-medical sciences. There are over 500 biomedical research institutes, most of which publish original papers in their collected works, or in conference proceedings. Selection, therefore, is a paramount problem.

But selection is made difficult by the small number of Russian-speaking scientists who have kept up with the Soviet literature, by the relatively small acquaintance American scientists have with their Soviet colleagues, and by the generally small amount of literature available for examination and evaluation in the United States. Previous Russian efforts to acquaint American scientists with their publications have been conducted through *Mezhdunarodnaya Kniga*, the state book export firm, whose values have been commercial and propagandistic, rather than scientific.

In the journal field, there are guidelines to be found in the frequency with which certain journals are cited in the international scientific

literature, in the quality of work done as evidenced by the number of individual papers translated and used, and the summary quality judgments made by those intimately acquainted with the field. Consultation with individual scientists, and the pooling of their judgments on the so-called top journals, was essentially the technique used in the selection of the eight journals whose cover-to-cover translation NIH is supporting.

There is, of course, the question of selecting the fields of research activity. In an area comprising as many disciplines and specialties as medicine, the support of translation in dermatology, for example, and not in dental medicine might prove inequitable. NIH solved this problem by selecting basic science journals (biochemistry, biophysics, microbiology, physiology) for cover-to-cover translation, and supplementing the cover-to-cover translation with a more highly selective plan for the many medical specialties.

Advance copies of *Abstracts of Soviet Medicine*, published by Excerpta Medica, are sent to 37 editors of American specialty journals who have signified their interest in the program. In accordance with their own editorial policies the editors select papers for translation, and possible republication. Papers thus selected must withstand competition for space with Western contributions, since NIH supports the translation, but not the publication.

The program emphasizes the translation of original research papers; the broad aspects of the organization and practice of medicine in the Soviet Union have not been included. It may be argued that it is not possible to evaluate Russian medical research without a knowledge of its traditional trends and of its sociological backgrounds. While these would doubtless prove a profitable field for academic investigation, the needs for reporting significant Russian progress are immediate, and the decision to limit the program to the reporting of research accomplishments was a pragmatic one. The program has different interests, therefore, from those which gave rise to the wartime *American Review of Soviet Medicine*, and the currently published *Review of Eastern Medical Sciences*.

The selection of abstracts for publication represents a compromise solution. From 1948 until 1957 the Academy of Medical Sciences sponsored the publication of a sixteen-section *Sovetskoe Meditsinskoe Referativnoe Obozrenie* (Soviet Medical Abstract Review) which, unlike the abstract journals of the Academy of Sciences, contained Russian contributions only. In that this publication abstracted the *Trudy* of Institutes and dissertations as well as journals, it was a most comprehensive service. After the Public Health Service had given a grant to Excerpta Medica for the translation of sections of SMRO, the Russians announced a change in publication plans. The Academy of Medical Sciences is publishing medical abstracts as of January 1, 1957 in four monthly abstract journals under the title of *Meditsinskie Referativnye Zhurnaly*.

Excerpta Medica had previously secured the cooperation of the Presidium of the Academy of Medical Sciences in naming thirty advisory editors under the chairmanship of Prof. A. V. Lebedinskii. The abstracts contributed by these editors, supplemented by additional ones translated from the new Russian abstract journal, totalling in all approximately 4,000 annually, form the basis of selection for the English language *Abstracts of Soviet Medicine*.

The selection of monographs for translation constitutes a difficult problem. There is little to be gained directly from the translation of popularizations or textbooks. On the other hand, considerable original research is published in the form of 60- to 90-page bound pamphlets. The problem is to identify those titles which contain original or suggestive work whose reissuance would stimulate investigation. By both Russian and world standards the work must be dependable, and there must be an expressed or latent need.

To meet the problem of monograph selection, the National Institutes of Health established an ad hoc Advisory Panel under the chairmanship of Dr. Michael B. Shimkin. This panel has met, and has made a number of selections. NIH will welcome suggestions of other titles which will meet its criteria.

Problems of Form. Traditional scientific communication systems depend on a heirarchy of journals, abstracts and indexes, reviews, monographs and textbooks, and finally encylopedic works. Russian publication follows this basic pattern. Where practicable, the NIH program has attempted to reproduce those forms which best convey information about research.

The desirability of cover-to-cover journal translations is occasionally questioned. The points are made that Russian journal articles are frequently irregular in quality, and that only infrequently does the individual investigator find

papers of significance to him. These points are equally true of journals published in English or any other language. Quality of papers is a function of editorial acumen and integrity. By accident of their evolutionary development, journals are polygraphic, and not necessarily the most efficient mechanisms for scientific communication. If the scientific standing and integrity of the Russian editors is a known quantity, there is no reason why a Russian journal in translation is a less effective instrument than an American one. The composition of the editorial boards for the eight journals supported under the NIH program was carefully reviewed.

The translation of individual papers at the request of editors of American specialty journals has already been described. In addition, the Pergamon Institute, the publisher of six of the eight NIH-sponsored journals, publishes in some of the journals a comprehensive bibliography of all contributions currently published in the field. The Institute offers translation service at reasonable rates for the papers so listed. The NIH program of republication of papers selected by American editors together with the Pergamon Institute program for demand translations from journals operate to insure a fairly comprehensive coverage of scientific contributions appearing in journal form.

Secondary publication, or the analytical indexing and abstracting of journal publication, is of course significant, in that it represents the first step toward the subject synthesis of scattered publication. The Russian pre-War index *Meditsinskaya Literatura S.S.S.R* has been brought up to date by two supplements (6–7), but in both comprehensiveness and fullness of information the abstract publications *Sovetskoe Meditsinskoe Referativnoe Obozrenie* (1948–56) and the *Meditsinskie Referativnye Zhurnaly* (1957–), previously discussed, have more to offer.

Excerpta Medica's translated publication *Abstracts of Soviet Medicine* varies from the standared Excerpta Medica pattern in that its categorization is by national origin, rather than by subject. There are immediate advantages in thus offering the totality of Russian medical publication. On the other hand, there are long-term advantages in absorbing the Russian national product into a universal international system arranged by subject. However the question of form of publication is ultimately resolved, it is safe to predict that workers in the medical sciences will be supplied with more abstracts from the Russian literature than have heretofore been available.

Considered as a publication form, review papers represent the first effort to evaluate and synthesize scattered publication. They offer not only a survey of the status and trends of research activity in a given field, but also summary judgments by professionally competent individuals on the significance of individual contributions.

In communicating Russian science, review papers have a particularly important role to play. The literature is so voluminous that it is necessary to depend on the reviewer's skill for selection, interpretation and pre-digestion. Editors report that it is difficult to find qualified individuals with the time to write reviews of the Western literature. With Russian literature this difficulty is compounded by the added requirements of a knowledge of Russian research developments and the requisite language skills.

The National Science Foundation has supported the preparation and publication of a number of comprehensive reviews of Russian scientific accomplishment in the *Annual Reviews* series.

Under the Public Health Service program of supporting investigations proposed and initiated by research scientists throughout the country, applications for grants to review Russian scientific literature are necessarily uncoordinated. The approach differs, for example, the type of planned survey of Russian science conducted by the East European Institute of the Free University in Berlin.

During this past year the Public Health Service has made grants for reviewing the Russian literature in the field of cardiovascular pathology, of neurochemistry, and of air pollution. The identification of areas for evaluation and review, and the stimulation of applications from individuals with proven linguistic and scientific ability remains a continuing problem.

Problems of Distribution. The Senate's mandate to make the full findings of Russian research "available" to American scientists is compatible with the Public Health Service Act which authorized the Service "to collect and make available through publications and other appropriate means information as to, and the practical application of, such research."

Dissemination of information lies at the heart of the translation project. In general, the National

Institutes of Health has followed a two-fold distribution pattern. The first objective has been to make the program's publications freely available to research scientists through the libraries of the institutions in which the bulk of medical research activity is conducted. The second has been to make the publications available to all interested individuals at a cost comparable to that of domestic scientific publications.

The National Institutes of Health sought the assistance of the Medical Library Association in the development of a primary distribution list of 300 medical and scientific libraries. It then supplemented this list by adding governmental medical research institutions proposed by the Surgeons General of the Army, Navy and Air Force, and the Chief Medical Officer of the Veterans Administration. The total distribution list approximates 400 institutions. The libraries are those of tax-supported or non-profit institutions. An underlying consideration is the fact that these institutions are those in which the bulk of the medical research conducted under Federal government grants is performed. By making this specialized information available in these institutions, the program hopes to provide added support for work done under Federal grants.

A second objective is to offer these publications to a wider market, both here and abroad, at a reasonable price. Where parts of the program are conducted by contract, this is so specified. For example, the Consultants Bureau reduced the price of *Biochemistry* from $95.00 to $20.00 in consideration of support received from the Public Health Service, and the journals issued by the Pergamon Institute are available at prices approximating their cost of production. This same principle will hold true for other publications sponsored by the program.

Problems of Acceptability. The ultimate success of the Russian translation program can of course only be measured by the degree to which Russian research findings are incorporated into Western scientific thought. This degree is purely and entirely a matter for the American scientific community to determine on the basis of repeated cross-checking of the experimental results reported. The function of the program is limited to making these results available for scrutiny and independent verification. The problem of doing this effectively involves not only selection and distribution, but also form of presentation. For example, muddy photomicrographs of pathological tissue would be meaningless to an American scientific audience conditioned to expect top quality reproductions in American journals.

Care has been taken to present the Russian translations in a form which might assure maximum acceptability by the scientific community. Where possible, letterpress on good quality paper has been preferred as more nearly approximating the standard of publication expected by medical scientists. Similarly, Russian cooperation with contractors has made possible the reproduction of half tone illustrations from original glossy photographic prints. By maintaining a standard of publication comparable to that of Western journals, the National Institutes of Health hope to remove any mechanical barriers which might impede the objective scientific evaluation of the Russian research papers.

The Question of Translation Rights. The United States and the U.S.S.R. are not signatory in common to any copyright agreement. There is, therefore, no legal impediment to the Russian translation of American scientific books and journals or to American translation of the Russian.

The U.S.S.R. Ministry of Foreign Trade has shown an interest in exploiting the commercial possibilities of translation from the Russian, but since in general this activity is not commercially profitable and must be conducted with the support of government or private foundations, the Ministry has made little progress.

From the very beginning, the National Institutes of Health has viewed the translation program as one to be conducted on a level of international communication among scientists in which the gains to be made are scientific and not commercial. The National Institutes of Health has therefore kept the Presidium of the Academy of Medical Sciences of the U.S.S.R. informed of its intentions.

The experience of scientific visitors to the Soviet Union has shown time and again that Russian scientists are most anxious to have their work known and appreciated in Western countries, and the climate for the continuation of the program on the level of free and unencumbered exchange of scientific information appears favorable.

APPENDIX

Outline of NIH Russian Scientific Translation Program

1) Journal Translations. The following journals have been translated and republished:

*Biochemistry**	$20 a year
*Bulletin of Experimental Biology and Medicine**	$20 a year
Biophysics†	$30 a year
Journal of Microbiology, Epidemiology and Immunobiology†	$50 a year
Problems of Oncology†	$30 a year
Problems of Virology†	$20 a year
Sechenov Physiological Journal of the U.S.S.R.†	$45 a year
Problems of Hematology and Blood Transfusion†	$20 a year

2) Individual Journal Papers. Thirty-seven editors of American specialty journals have agreed to scan Soviet abstracts in their fields for the purpose of selecting Russian research papers for translation, editing, and potential republication.

3) Abstracts. Under grant from the Public Health Service, Excerpta Medica is publishing approximately 4,000 translated abstracts annually in *Abstracts of Soviet Medicine*. This journal has two parts: *A*, devoted to the basic medical sciences; and *B*, to clinical medicine, both issued quarterly. *Abstracts of Soviet Medicine* is distributed under the program, and is also available for subscription at $15.00 per section or $25.00 for both.

4) Russian-English Medical Dictionary. A 27,450-word dictionary has been compiled by Stanley Jablonski of the National Library of Medicine and edited by Dr. Benjamin S. Levine. The dictionary is scheduled for publication in the spring of 1958.

5) Directory of Soviet Research Institutes. A preliminary edition of a directory of nearly 500 research institutes in the fields of biology and medicine is available on request from the Library, National Institutes of Health.

6) Grant to Translation Center, John Crerar Library, Chicago. The National Institutes of Health is cooperating with the National Science Foundation in the support of a national pool of scientific and technical translations maintained by the Special Libraries Association at the John Crerar Library, Chicago. This Center publishes a listing of all translations received under the title of *Translations Monthly* (subscription price: $5.00 per year).

7) Translation of Selected Monographs. The National Institutes of Health is supporting the translation and publication of specialized monographic literature screened by an ad hoc Advisory Panel of scientists and selected on the basis of originality and stimulus to American research activity.

8) Review Papers. The National Institutes of Health is accepting applications for research grants to support the preparation of critical reviews of segments of Soviet research accomplishments.

9 Miscellaneous. The Program has supported the publication by the Public Health Service of *Ticks or Ixodides of the U.S.S.R.: A Review of the Literature*, by Dr. George Anastos, University of Maryland, and of *The United States-U.S.S.R. Medical Exchange Missions on Microbiology and Epidemiology—1956*. A digest of comments made by American scientific visitors to the Soviet Union entitled *Medical Research in the Soviet Union*, and copies of *Scientific Translations: A Preliminary Guide to Sources and Services*, prepared by the National Library of Medicine, are available from the Library, National Institutes of Health, on request.

* Published by Consultants Bureau, New York.

† Published by Pergamon Institute of New York and London.

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CODING OF A SMALL REPRINT COLLECTION ON THE CENTRAL NERVOUS SYSTEM

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MANY scientists who have about 5,000 reprints in current use feel that the various punch card systems are too complex and involved to be worth using for such a small group. We are presenting this report in hopes of demonstrating that such collections may be easily dealt with by means of Keysort cards, provided that both the scientist who wishes to use the system and his secretary, librarian, or technical assistant agree on the following points: *1*) the basic purposes which the system is to fulfill; *2*) the advantages and disadvantages of any given plan; and *3*) a critical evaluation of these advantages and disadvantages. This paper is not presented as a discussion of coding *per se*, but as a description of the problems that must be answered in dealing with a small collection.

Before discussing the principles used it would be worth while to review our particular research organization. The group consists of ten to twelve principal investigators under a research director. All problems are basically related to the brain and are either neurochemical, neurophysiological, neuropharmacological, or clinical. The basic purpose of all research is to obtain information on brain tissue which will help to elucidate clinical conditions referable to the central nervous system.

The purpose and the nature of the group interested in the reprints have made it possible to limit rather arbitrarily the scope of the collection to be coded. For example, only those aspects of subjects such as *Alcoholism* which refer to the brain are considered; that is, the social and psychological aspects of the problem would not be covered. In the same way, papers on cerebral palsy are included only when they contain data concerning the actual biochemical and physiological state of the central nervous system in this disease. The ability to restrict the scope of the problem in these ways makes, of course, for a much simpler coding system than would have been possible under other circumstances. The procedure for dealing with out-of-scope reprints will be discussed later.

Our coding has been modified considerably over the 5-year period it has been in use. At the time that the laboratory opened, it was decided to make a Keysort card for each of the reprints in the Director's collection, typing on the face of each card the complete reference. It was hoped that eventually the cards would encompass the entire reprint collection of the laboratory personnel. However, this has not been done to date, partly because of the inevitable turnover of personnel. At that time—5 years ago—the collection consisted largely of papers which pertained to previous work carried out by the Director in other laboratories or to problems which it was felt might be undertaken in the new laboratory. The cards were set up without a detailed subject code but with author, journal title, volume and year punched on the card. About 14 months ago, the entire system was reevaluated and a new system, including detailed subject coding, was developed. This overhauling was necessary partly because, in the period of 5 years, the situation changed from that of a newly started laboratory to one with a number of integrated, continuing programs and for that reason the ideas of what reprints were important also changed.

In setting up the coding system, we decided that reprints would be filed arbitrarily by number, and the cards themselves alphabetically by the name of the principal author. The principal author is considered to be the one whose name is associated in the minds of scientists with the specific research problem. The year was retained as a punch on the card, and the rest of the card saved for subject coding.

So far, with the exception of determining the name of the principal author the work could be done by the non-technical staff. The plan for subject coding was then developed to meet the following criteria:

1) The basic subject code developed through consultation with the various scientists should be such that the non-scientific staff could use it without constant consultation with the scientists.

FIG. 1. Completed Keysort card.

2) False sorts were not eliminated or even kept to a minimum. It was felt that less time would be spent by a scientist sorting through 100 cards, only 2 of which might meet his requirements exactly, than to construct a code of such detail and precision that false sorts would be eliminated. A scientist often can tell simply by scanning titles under a broad subject which papers will contain the specific information that he needs. In addition, this broad scanning will serve the same function as browsing in *Biological Abstracts* or other indexing or abstracting tools.

3) On the face of the card the *specific* subject for which the article is coded should be indicated (fig. 1).

4) The code arbitrarily omits certain groups of papers or types of coding. Papers dealing primarily with chemical methods would be kept out of this coding group. If the time ever comes that the collection of such papers is large enough, a separate file would be prepared for them. The same is true of other out-of-scope papers.

5) An alphabetical index to the code, including all terms and synonyms, should be available at all times for purposes of searching and coding.

6) Highly classified indices arranged on as many axes as possible should be developed by consultation with the scientific staff. New subjects coded should be periodically scanned and added to the classified index.

7) Preliminary sorting in most cases should be done by the non-technical staff upon request by the scientists.

When the list of subjects to be coded was compiled it was agreed that the code was to be designed for current use, and that within approximately 5 years many of the reprints would no longer be of much interest. We plan at the end of 5 years to reevaluate the entire collection, to withdraw the newer reprints and any others which are still of interest as a nucleus around which to construct a new code. The older reprints would then form an inactive file, which would remain available for another 5 years. While this entails a considerable amount of work every 5 years it will keep the size of the collection under the maximum for which we feel this type of code is most efficient. It also eliminates the necessity for extensive modification of the current code as our interests change.

With these considerations in mind it was possible to construct a very simple code for a restricted list of subjects. The concepts to be coded were grouped in nine categories: *1*) Parts of the brain, *2*) Topics of current interest, *3*) Experimental methods, *4*) Age, *5*) Animals, *6*) Function of chemical constituents, e.g. neurohormone, coenzyme, etc., *7*) Drugs, *8*) Chemical constituents, *9*) Pathological states. Field numbers on the card correspond to the category numbers (fig. 2). In addition, one field was used to designate the year of publication, and the

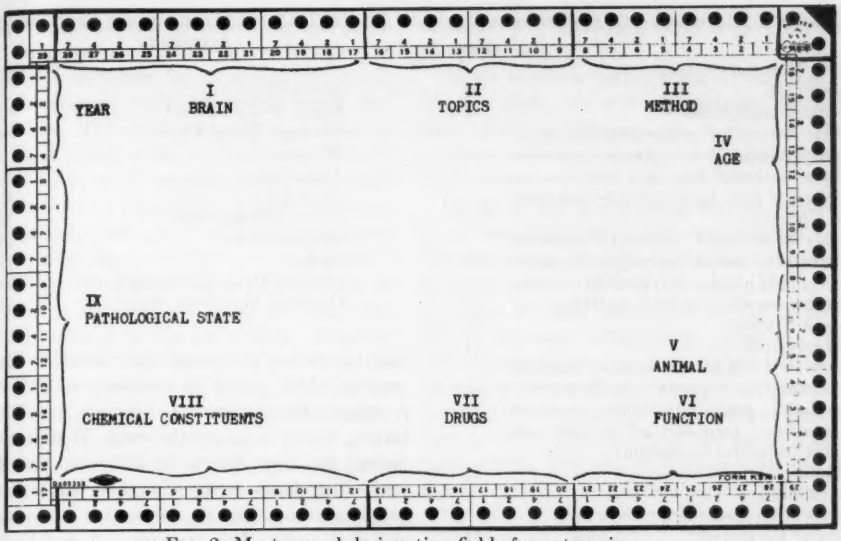

FIG. 2. Master card designating fields for categories.

corner spaces were reserved to indicate papers which might be of long range interest. Within these categories the terms were listed at random and code numbers assigned without any attempt at classification. Therefore, considerable expansion may be made without any modification of the present code by adding terms to the unused numbers left in each category.

Through the use of this kind of categorization it is possible to consider any concept in relation to any other concept in one or more of the categories. If all papers on the use of psychopharmacological agents in psychoses were desired, two sorts would be necessary, one for psychopharmacological agents and one for psychoses. If only those dealing with psychopharmacological agents in psychotic children were desired, a further sort would be made in the category for age. Similarly, any chemical constituent might be considered as it related to a part of the brain or a pathological state.

In the category for *Parts of the brain*, our selection of terms to be coded was a very personal one and determined more by our interests in certain structures than by a need to cover all anatomical structures (table 1). For instance, because of our interest in the blood-brain barrier it seemed desirable to group together the areas usually considered to be outside the blood-brain barrier, namely, area postrema, tuber cinereum and choroid plexus, and give them a single code number. Our particular problems in the same way made it possible to restrict the code for *Chemical constituents* of the brain to those for which there were determinations in the literature and those on which work was likely to be done in the near future in our laboratory. The use of *Function* coding makes it possible to group the chemical constituents as enzymes, neuro-hormones, antimetabolites, etc. Usually both of these fields are to be punched, but in some cases only one field would be used. For example, if an antimetabolite such as 3-indole-acetic acid is fed, the indole-acetic acid would not be punched, but the code number for antimetabolite would be. Similarly, a constituent such as water would not be punched for function.

The codes for *Animals* and for *Age* reflect our special interest in comparative neurology and neurochemistry on one hand, and in the chemical changes which occur in the brain as it develops, matures and ages, on the other. This information is seldom indicated adequately in the title, and must be unearthed from the fine print.

The code of *Experimental method* includes those which we are currently using in the laboratory, or plan to use; for example, electroencephalography, production of brain lesions by x-irradiation, convulsions, hypoglycemia, etc.

The section devoted to *Topics* is designed to pull together papers dealing with broad aspects of problems such as cerebral blood flow, metabolism, behavior, and neurophylogenesis (table 2). *Pathological states*, for example, hypothermia,

TABLE 1. CATEGORY 1—PARTS OF BRAIN

	Punch
Central Nervous System	1
Whole Brain	2
Cerebral Hemispheres	4
Terms to be coded: cortex, cortical gray, gray matter, white matter, parietal lobe, occipital lobe and pole, frontal lobe and pole, temporal lobe and pole.	
Basal Ganglia	7
Terms to be coded: lenticular nucleus, putamen, neopallidum globus, pallidus, pallidum, amygdaloid, claustrum; see also (caudate nucleus)	
Caudate Nucleus	10
Mesencephalon	11
Terms to be coded: midbrain, cerebral peduncles, corpora quadrigemina, colliculi, geniculate bodies, cerebral aqueduct, aqueduct of sylvius; see also (reticular formation)	
Thalamus	12
Hypothalmus	
Terms to be coded: mammillary body	
Reticular Formation	17
Terms to be coded: mesodiencephalic activating system, activating system	
Cerebellum	20
Terms to be coded: lateral lobes, vermis, paraflocculis, median lobe.	
Medulla	21
Rhinencephalon	22
Terms to be coded: gyrus fornicatus, hippocampus, limbic lobe, archipallium; see also (amygdaloid nucleus)	
Areas Outside Blood-Brain Barrier	24
Terms to be coded: tuber cinereum, area postrema, choroid plexus	
Spinal Cord	27
Cerebro-Spinal Fluid	40
Nerve Preparations	41
Terms to be coded: isolated nerve	
Glia	42
Terms to be coded: microglia, neuroglia, oligodendroglia, astrocytes	
Neurones	44
Terms to be coded: dendrites, axons, cell body	
Cerebral Blood Vessels	47
Terms to be coded: sinuses, arteries, veins	
Corpus Callosum	70

TABLE 2. CATEGORY 2—TOPICS OF CURRENT INTEREST

	Punch
Metabolism	1
Blood-Brain Barrier	2
Cerebral Blood Flow	4
Behavior	7
Activation	10
Blocking	11
Neurophylogenesis	12
Detoxication	14
Aging	17
Clinical Drug Evaluation	20
Cerebral Metabolic Rate	21

hypoglycemia, mental deficiency and psychosis, are punched in one field.

The code devoted to *Drugs* presented somewhat different problems. Even a restricted list of specific drugs which would be of importance to us was found to contain many more terms than could be coded in the space allotted on the card without using a more complicated numerical code than we considered desirable. Because the rapid development of new drugs makes it difficult to predict the extent and direction of expansion which would be necessary in the code, it seemed impracticable to provide for this by leaving blank areas on the card. Therefore the desired flexibility had to be built into the code. Only drug classes were coded, and the code was supplemented with an exhaustive and highly classified index. For example, all psychopharmacological drugs could be indicated by a single punch on the card, while the index would indicate all drugs to be coded in this manner. Such a scheme, of course, means that a search for chlorpromazine would require some hand sorting to eliminate all irrelevant cards. As an aid to sorting for the particular cards desired, the names of the specific drugs discussed in the paper are shown on the face of the Keysort card. These lists give detailed information not always apparent in the title of the paper and make it unnecessary to scan papers not germane to the problem. Since in most instances a search would be made for chlorpromazine as it related to one or more other concepts, the tendency is to reduce the number of cards to be sorted by hand to manageable proportions. The plan also has the advantage that papers discussing several psychopharmacological agents can be indicated by a single code number.

The index as well as the code were set up with the help of a scientifically trained person. In many instances it was necessary to be somewhat arbitrary in assigning a particular term to one group or another; that is, lysergic acid diethylamide might be classified as a sympathomimetic drug or as a psychomimetic drug. Since its role as a psychomimetic was of primary interest in our laboratory it was classified as such and a cross reference made from sympathomimetic. Such arbitrary decisions create no real problem as long as each concept is classified in only one

way and sufficient cross references are used in the index. In a case such as the above it would be necessary in a search for all sympathomimetic drugs to make an additional search under psychomimetics. The need for additional searches would be indicated in the classified index. Since a search is made much more frequently for a specific drug than for all drugs of a particular class, the additional work involved in searching for a complete class does not seem to be a burden.

The alphabetical index was set up on cards so that as new materials came to our attention they could be assigned to the appropriate categories and the necessary information inserted in the index. The terms used were then grouped on as many axes as possible for the classified index. For example, glutamic acid was classified as an amino acid, as an ammonia trapping agent, as a hyperglycemic agent, etc. Much of the value of the system depends upon our ability to keep an up-to-date classified index which expands as our ideas and interests change. We feel that it is especially important that as new problems are developed and as new personnel join our group, the classification in the index be changed to include their interests. No work will be involved on the code itself other than the addition of new subjects, but the indexes will require constant reorganization.

As we continue through the 5-year limit set for this code, we will undoubtedly encounter many problems we have not considered. These problems will be dealt with on the next revision of the code.

We would like to emphasize that this approach is an exceedingly pragmatic one designed to fit one definite set of conditions. It does not attempt to follow any one theory of coding and has only one aim—to make information readily available with a minimum waste of the scientist's time. The scheme is given in more or less detail, not because we feel that others may wish to copy it, but simply as an example of how simple a system may be which combines both the use of a scientist's normal browsing techniques with the efficiency of a Keysort or other punch system.

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A UNIQUE SYSTEM FOR RAPID ACCESS TO LARGE VOLUMES OF PHARMACOLOGICAL DATA; APPLICATION TO PUBLISHED LITERATURE ON CHLORPROMAZINE

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IT HAS become increasingly evident in recent years that one of the major problems in the efficient use of scientific manpower is the need for easy and complete access to reports of work already done. Recognition of this need has led to the growth of documentation as a special field of science. Special techniques have had to be developed for the storage of large volumes of information and the rapid retrieval of this information when it is required by scientists. We would like to describe one of the techniques which the Science Information Department of Smith, Kline and French Laboratories has developed to handle pharmacological information.

Anyone who has worked with a new drug knows that there is usually a frustrating lack of information about it. When Smith, Kline and French became interested in chlorpromazine, we were faced with an unusual problem. We were presented with a large volume of information about the drug, which grew larger every week and soon threatened to swamp our facilities for keeping track of it. Long before we were ready to market Thorazine, it became apparent that we would have to devote our whole Science Information Department to this one drug if we did not find better ways of handling the data on it.

There are a number of well-known ways of handling an accumulation of reports and reprints.

1) They can be filed according to subject, either alphabetically or according to a numerical coding system. This works only with a small collection and only if authors have cooperated by discussing just one subject in each report.

2) They can be filed by date of receipt, accession number, author's name, or some other neutral identifier. In this case, a separate card index is often used to supplement the report file. This index can be as specific or as broad as the owner chooses. Card indexing will handle a considerably larger collection than subject filing of the original reports, and allows more thorough indexing of each document. It becomes unmanageable when the total number of index entries desired is high. In addition, it cannot easily identify a document that discusses more than one subject.

3) When a card index, with separate cards for each subject in each document, becomes too large for efficient use, some sort of a unit record is necessary. A unit record system involves a recording medium, such as punched cards, which still permits multiple indexing but requires only one card for each document. Punched cards can be searched either manually, using edge-punched cards, or by machine. Manual searching is inexpensive and is efficient for files up to about a thousand documents, depending upon the complexity of the searches required. Beyond this, machines become necessary. A primary characteristic of punched card systems is that all subject headings for a given document can usually be entered on one card; thus the card file contains only as many cards as there are documents. This obviously results in a considerable saving of filing space and time compared to a conventional card index. To be sure, in such a system, every card must be examined during each search. This basic disadvantage is offset, particularly in machine searching, by the speed of the machine and its ability to select simultaneously the cards which meet a complex set of characteristics.

The flood of information on chlorpromazine forced us to re-examine our methods for handling such data. Simple subject filing obviously could not handle several thousand detailed documents. A card index was a useful stop-gap, but it could not cope with the tide for very long. More important, it could not be used for correlating and tabulating data. An estimate of the amount of data we might expect to accumulate within a few years was enough to convince us that machine-sorted punched cards were the only way to keep the situation under control.

The wisdom of this decision soon became apparent. We now have more than 10,000 documents, published and unpublished, in our chlor-

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promazine file. These documents can be divided into clinical and pharmacological reports, although there is considerable overlap. Since the needs of the clinicians and the pharmacologists for recall of data differ, these two types of information are handled somewhat differently, and only the pharmacological system will be discussed here. It should be pointed out, however, that all the data, clinical or pharmacological, in any one document are entered on one card, so that correlation between the two can be made if desired.

We think the system we have worked out for storing and retrieving pharmacological information is quite simple. It has to be, since machine documentation is only one of the activities of our busy Science Information Department. As with any punched card system, it is based on three essential factors: *1*) an index, or set of subject headings; *2*) a means of transferring this index to a pattern of holes in a punched card; and *3*) a machine which will examine these punched cards and sort out the ones which contain the desired subject headings. Each of these factors is discussed in detail below.

Anyone who has ever tried to index a file of reprints or abstracts knows that the first essential is a list of subject headings. The larger the file, the more necessary it is to have a comprehensive list that, as far as possible, anticipates all questions that might some day be asked of the file. A hundred documents can be re-indexed if the collector's point of view changes; 500 make the job pretty difficult, and 1000 make it impossible. We anticipated that the chlorpromazine pharmacology file might eventually grow to several thousand documents. Therefore, our first job was to set up a subject heading list or index that would include every concept we might ever need to use to describe their contents. The list should be specific enough to take care of all the chlorpromazine data, but it should also be general enough to apply to any drug. We believe our solution is unique and, at the same time, very simple.

The essential part of our subject heading list is a group of about 150 words or terms referring to organs, tissues or functions, and hormonal activities. This list includes almost any possible site of action. For convenience only, these terms are gathered into groups corresponding to body systems. For example, under central nervous system, we have included gross anatomical divisions like cerebrum, hypothalamus and spinal cord, and specific function areas like appetite regulation, emetic center and spinal reflexes. Any document containing information about the emetic or antiemetic action of a drug would be indexed under 'emetic center.' If it also contained information on inhibition of the patellar reflex, it would also be indexed under 'spinal reflexes.' However, simply knowing that there is an effect on the emetic center may not be specific enough. Was the study done in dogs or humans? Was the effect antagonism to apomorphine or motion sickness, or perhaps a study of enzyme systems of the emetic center itself? Was the effect observed only with very high doses? These questions are answered by a second set of terms, or descriptors, any of which can be applied to any word in the main anatomical list. There are words like dog, rodent and human to describe the test subject; words like unusual dose, toxicity and isotope study to describe special circumstances. The really distinctive feature of this system is a group of words describing the actual effect observed, such as action of the drug under consideration (called the *reference drug*) on function, metabolism, or histology of the tissue involved, effect of the reference drug on action or metabolism of some other drug or physical agent.

A combination of these descriptors with the site-of-action words will describe the contents of any pharmacology report. Thus, a paper reporting that very high doses of the *p*-chloro derivative of Boppo antagonize the emetic effect of apomorphine in dogs would be described by the terms: *Site of action:* emetic center; *descriptors:* effect of the reference drug on the action of another drug—dogs—unusual dose—derivative of the reference drug (Boppo).

A paper describing the effect of chlorpromazine on enzyme activity in liver slices would be indexed as: *Sites of action:* liver—tissue enzymes and metabolism; *descriptors:* effect of the reference drug (chlorpromazine) on metabolism—*in vitro* study.

Two years of experience have demonstrated that this subject heading list fills our specifications. It is comprehensive enough to cover any type of drug effect and it is general enough to be applied to any area of pharmacology or physiology. Since each word in it is independent of all the others, it can be expanded and extended at any time.

A carefully planned subject heading list is the most important part of any indexing system. It is the one aspect which absolutely requires the

efforts of a scientist familiar with the field of knowledge involved. Once this list has been prepared, reducing it to punched card codes becomes a mechanical problem. An IBM card has 80 vertical columns numbered from 1 to 80. Each of these columns has 12 punching positions. Every one of the total of 960 positions can be assigned a particular meaning. In normal punched card procedures, only one punching position in any one column should be used at one time. Therefore, each column is usually used to record one piece of information. For example, column 10 could be assigned to species, with position 0 indicating dogs; 1, cats; 2, rats; and so on. A punch in the 11th or so-called x-position of the column might mean that several species are mentioned. Numerical information may be entered as such. For example, columns 18–20 might be used to record dose in mg/kg. In that case, 125 mg/kg would be punched as column 18, position 1, column 19/2, and 20/5. The identification number of the document is often punched in the first or last 6 columns of the card.

Since there are 12 punching positions in each column, our list of less than 260 index headings would require approximately 18 columns of assigned positions, or direct punches. However, very few of these items would be needed to describe any one document, and most of the 18 columns would be blank on every card. Since the same card also had to contain clinical data, document number, author identification, and other general information, all available space had to be used as efficiently as possible. If, in the future, the subject heading list should be expanded, it would require still more space, which would probably not be available. These considerations called for some modification of the conventional methods.

As mentioned above, normal punching procedure does not permit unplanned use of more than one punch in a column. However, it is quite legitimate to plan to use a specified number of punches in each column. If we assign a specific meaning not to each punch in a column but to each combination of punches, the number of possible entries is greatly increased. If, instead of saying that a punch in position 0 indicates dogs, 1 means cats, and so on, we say that the combination of 0 and 1 in the same column is spaniels, 0–2 is beagles, 0–3 is boxers, 1–2 is alley cats, x-9 is human beings, and so on, we now have something like 66 things to choose from instead of 12. If a combination of 6 punches in a column is used, there are 924 possible combinations, that is, 924 possible entries, any one of which can go in one column. If each of the 924 items had been assigned one specific position, we would have had to use 77 of the 80 columns on the card, since each item must always appear in the same place. Using 6-digit codes, we can put any item in any column. We therefore need to reserve only a few columns, enough to contain the maximum number of terms to describe one document.

Our system for storing pharmacological information actually uses both types of punching. We have 4 columns of assigned positions or direct punches for the 48 descriptors, the words that apply to any study such as species, type of action, and special circumstances, since we need to use some of these for every document. We use the 6-digit codes for the site-of-action headings, the anatomical terms. We have found that we seldom need more than 3 or 4 of these terms for any document, but to be safe, we have allowed 7 columns. Remember that any of as many as 924 subjects can go in any of these 7 columns. One additional column of direct punches for body systems has been added, to take care of requests for all documents describing any effect of chlorpromazine on the cardiovascular system, for example. (See APPENDIX for sample portions of the finished code.)

This code sheet containing the subject headings and the codes assigned to them takes care of the input of the system. Entering information in the file requires only two people, a coder and a key-punch operator. Operation of input can be described by following the paper mentioned above, on the antiapomorphine action of *p*-chloro-Boppo. The coder reads the paper and decides which of the code words apply. She circles these words and their accompanying numbers on a code sheet, stamps the sheet 'Boppo,' and passes code sheet and original document on to the key-punch operator. If this is document number 11,539 in the Boppo file, the first 6 columns of the card will be punched 011539. On our cards, columns 7 through 34 are reserved for clinical data, but since the document in question is a pharmacology paper they are left blank. Position 3 in column 35 is punched to indicate that the study involved dogs. Column 36, position 1 indicates that an unusual dose was used; 37/3, that a derivative of Boppo was involved; 38/3, a central nervous system effect, and 39/3, an effect on the action of another drug. Columns 40-

46 are reserved for the site-of-action codes. In this case, the 6-digit code for emetic center (013-689) goes in column 40; the others are left blank. Other punches on the card will indicate that this is a published paper by Jones from the University of Minnesota and that it contains pharmacological data.

The coding of a report on the antihistaminic effect of chlorpromazine in isolated preparations of guinea pig small intestine and tracheal chain will illustrate how a more complex situation is handled. We have made the somewhat arbitrary decision that histamine should be considered a type of hormone and therefore should be included among the main subject headings. Therefore, our 6-digit codes might be column 40/y34689 for histamine, column 41/145678 for small intestine, and column 42/023569 for trachea. The order in which these are entered is unimportant. Any of the three can be in any of those three columns. Since histamine is a main subject heading, column 39 is punched in position 0, effect of chlorpromazine on function, function in this case being the action of histamine. The other descriptor codes are position 7 in column 35 to indicate *in vitro* study, and position 4 in column 38 to indicate the hormones, since histamine is considered to be the main subject of the paper. It should be stressed that the 6-digit codes are not essential to the operation of the system. They are merely a convenient device which allows us to tie up only a small part of the card, while maintaining a very large reservoir of index entries from which to choose.

These examples illustrate the use of the code to prepare our reference file. The machine we use to search this file is IBM's Model 101 Electronic Statistical Machine. This machine was designed originally to handle census information, and is a big brother to the sorter that picks out the questions on cooking or Abraham Lincoln on the television quiz program. It is not a computer or a giant brain. All it can do is sort and count. Unlike its little brother, it can also do a limited amount of printing and, most important, it can sort for several criteria at a time. The standard sorter can read and sort on only one column, that is, for one characteristic at a time. If you wish to look for a second factor, the cards must be run through a second time. The 101 reads all the columns on a card in one pass and can therefore be instructed to pick out cards which contain several criteria at once. Instructions for each search are given to the machine through a control panel wired by the operator. The wiring procedure is relatively simple, and need not be described here. The important thing is that the machine will separate from a large deck of cards those which contain any desired combination of characteristics. These characteristics include both the presence and the absence of certain punches; that is, we can sort out all cards which combine characteristics A, B, and C, or all cards which contain characteristics A and B only if C is not present. For example, we can obtain all cards for documents which report antagonism to the emetic action of drugs in dogs. We can also specify that they should not contain any reference to sedative effect.

These three factors—the subject heading list, the coding procedure and the machine—compose our system. When a request comes from one of our scientists for all the information on some aspect of chlorpromazine pharmacology, the supervisor checks the code sheet for the appropriate codes. The machine operator wires the control panel according to these codes, and runs the cards through the machine. Since the document number is typed on each of the cards, those which have been selected can be examined visually, or the machine can be instructed to produce a printed list of document numbers. The corresponding documents are taken from the file for detailed study by the original requestor, or for abstracting by someone in the Science Information Department. If the request was for a bibliography, perhaps to be sent to someone outside the company, this can be prepared automatically. This is done on the Flexowriter, an automatic electric typewriter controlled by perforated paper tapes. One of these tapes has been prepared for each reference in the chlorpromazine bibliography. To prepare the special bibliography, the tapes for the references selected on the 101 are fed into the Flexowriter which types them out rapidly and accurately. Once the tapes have been prepared, the references can be reproduced as many times as desired, without the necessity for further proofreading.

The details of the system as we have described them here were designed to fit our equipment and our needs, and are only a small part of a larger operation. The method could, however, be readily adapted to a much smaller operation and much less expensive equipment. The same type of code sheet could be readily adapted to edge-notched cards, such as McBee cards, to eliminate the machines altogether. The most

important part of the whole system, from the pharmacologist's point of view, is the basic philosophy used in designing the subject heading list; that is, the idea of a set of organ and tissue words combined with a second set of words that indicate whether the effect is on function, metabolism or structure of the organ or on the action of another agent on it.

There are several advantages of our system over other methods of indexing:

1) It requires no decisions by the indexer about the relative importance of various factors. Considering a paper on epinephrine reversal by chlorpromazine, no one has to decide whether the significant entry should be epinephrine, chlorpromazine, pressor response, hypertension, or adrenolytic action. All these facets of information are coded. No matter which is used as a search criterion, the paper will be found.

2) Once the subject heading list has been set up, highly trained scientists are not needed to operate the system. The indexers must have scientific training, of course, since they must be able to read reports intelligently and translate the authors' words into the code words we use. An occasional arbitrary decision as to where something should be coded has been necessary. These arbitrary decisions do not decrease the accuracy of coding or retrieval, since everyone who uses the system knows which concepts are included under each code word.

3) Uniformity of indexing is very high. We have tested this several times by having different people index the same papers and the results have always been very gratifying.

4) Relative speed of searching is inherent in machine systems. No time is lost deciding where to look for something or in hunting down 'see also' references. The IBM 101 examines cards at a rate of 450 per minute, or 9,000 in 20 minutes. Refiling of cards is unnecessary. They can be replaced in the file drawer in any order without regard to alphabetical or numerical arrangement. The only requirement is that they all be the same side up!

5) The subject heading list can be enlarged or made more detailed any time we think it is necessary without disturbing indexing already done, and without upsetting the entire classification system. If necessary, we can sort out cards referring to any particular subject and add more codes to them. The punched cards are very easily reproduced mechanically if extra decks are desired.

6) The code was planned to be applicable to any area of pharmacology or physiology. As a secondary benefit, we have found it useful for such things as indicating fields of interests of consultants, and even for describing side effects in patients.

This is, of course, a retrieval system. It does not give you the information you want, but it tells you which documents in the file contain it. We have found that this is the most satisfactory way to deal with pharmacological information. For handling clinical information, the code is set up with a high proportion of direct punches or assigned positions, so that we can also correlate and tabulate data. This is done by wiring the control panel to instruct the 101 to count the cards as it sorts them. The tables are then printed out by the machine just as we print out the document numbers.

It is important to realize that this type of index is not limited to the equipment which we use. It was designed for use with the IBM 101 sorter, but changing the code numbers, not the words, could readily adapt it to a simple sorter or to hand-sorted cards. It could probably also be adapted to a card index. Needless to say, it would also be applicable to other types of machine systems. The same philosophy could also be used in designing similar indexes for other fields, such as microbiology.

This unique system of indexing and searching pharmacological literature provides the scientist with several benefits. It frees him from many hours of tedious library searching. It also assures him of uniform, comprehensive analysis of all documents in his sphere of interest. At his request, he can obtain all the documents, and only the documents, which contain the information he desires. Such service, we feel, is an important factor in the most efficient and productive use of scientific manpower.

APPENDIX

Selected Portions of Pharmacology Code Sheet

Descriptors	Sites of Action
Column 35: Subject	*Central nervous system*
0 not specified, several	013456 cerebrum
1 rodents	013457 hypothalamus
2 birds, amphibia	013458 brain stem
3 dog and cat	013459 spinal cord
4 monkey	013578 temperature reg.
5 other animal	013579 appetite centers
6 human	013678 respiratory centers
7 *in vitro*	013679 vasomotor centers
8 ovum	013689 emetic centers
9 pregnant	014567 spinal reflexes
x fetus, newborn	*Gastrointestinal system*
y other	134789 stomach
Column 36: Special features	135678 gastric motility
0 unusual dose	135679 acid secretion
1 unusual route	135689 other secretion
9 absorption	135789 small intestine
x combinations	145678 s. i. motility
y comparisons	145679 s. i. absorption
Column 37: Type of study	145689 s. i. secretion
0 chemical	145789 colon
1 bioassay	146789 color absorption
2 pharmacy	156789 colon motility
3 derivative of RD*	*Hormonal actions*
4 isotope study RD	y12345 ACTH
5 isotope study OD†	y12346 TSH
y multiple action	y12347 STH
Column 38: Body system	y12348 other anterior pituitary
0 metabolism of RD	y12459 estrogens
1 blood, hemopoietic	y12467 progesterone
2 cardiovascular	y12468 other female
3 central nervous	y12469 androgens
4 endocrine, hormone	y12567 other male
5 gastrointestinal	y23456 adrenocortical
6 peripheral nervous	y23457 glucocorticoids
7 urogenital	y23458 cortisone
8 respiratory	y23459 hydrocortisone
9 bone, muscle, skin	y34678 adrenal medullary
x body in general	y34689 histamine
Column 39: Type of action	y34789 ACh
0 RD on function	
1 RD on metabolism	
2 RD on histology	
3 RD on action OD	
4 RD on metabolism OD	
5 RD on action PA‡	
6 OD on action RD	
7 OD on metabolism RD	
8 PA on action RD	
9 comparison with PA	

* RD—Reference drug.

† OD—Other drug. ‡ PA—Physical agent.

PROBLEMS OF CLASSIFICATION AND CODING IN THE BIOLOGICAL SCIENCES

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Webster's International Dictionary defines biological classification as "a systematic arrangement or method of arrangement of animals and plants in groups or categories according to some definite plan or sequence." Further, an accompanying definition of taxonomy reads "act of classifying; act of distributing into groups, as classes, families, etc., act of assigning to a proper class."

It is the purpose of this paper to point out that classification may be more broadly understood than these definitions might suggest, and that classification is of greater fundamental importance in natural science than existing classification systems might indicate.

Some time ago, John Stuart Mill observed that "as soon as we employ a name to connote attributes, the things, be they more or fewer, which happen to possess those attributes are constituted ipso facto a class." The essence of classification is to be sought, therefore, in the characterizing of things, devices, processes, etc.

The basic motivation in classification is to achieve useful groupings. The purposes thereby served may be highly diverse. For the moment, attention is directed to only two of the more important of such purposes.

The first is the evaluation of some newly observed entity or occurrence on the basis of past experience. For example, when we observe the characteristics of an organism, possibly pathogenic, and are able to accomplish its identification, we have classified it, at least in the sense of the above quotation from Mill. At the same time we have related our newly made observations concerning the organism to past experience, and thus made it possible to deal with it in terms of earlier knowledge. In this way, classification is an important tool for enabling—or at least facilitating—the application of previous knowledge to current problems.

A further highly important purpose served by classification is in the correlation of observations preparatory to establishing generalizations which, in the observational sciences, are often referred to as 'natural laws.' Here again, the classificatory step inevitably involves the characterization of observations on the basis of entities involved, processes occurring, measurements made, etc. In this connection, it is particularly important to keep in mind that our so-called natural laws are, after all, nothing more than our best attempts at correlating our observations. Constant improvement in such attempts is the hallmark of progress in science. At no stage in scientific development are we justified in assuming that the currently existing formulation of so-called 'natural laws' represents direct contact with ultimate truth.

This brief introductory discussion may suffice to make the following points. *1*) The broad purpose of classification is to serve human needs and requirements. These may be extremely diverse in nature and may range from such relatively abstract activity as the formulation of natural laws to the very practical purposes of diagnosis in medicine or the arrangement of kitchen utensils in a housewife's cupboard. *2*) The basis of all classification is the observation of certain characteristics. *3*) The act of classification is the establishment of groupings based on the characteristics as provided by observation.

Each of these three areas of classificatory activity is the source of various problems whose diverse nature may cause considerable perplexity and on occasion may even lead to deplorable misunderstanding and bickering.

It is scarcely possible within the confines of this paper to explore exhaustively, or even to list, all of the problems that may arise in connection with classification. We shall attempt to indicate some of the more important problems that have been observed to arise. Space will not permit a complete discussion and the reader is referred to Perry and Kent (1) for a more detailed description.

A Mathematical Model and Classification Procedures. It is sometimes overlooked that the very act of grouping—the establishment of a class or set of classes—presents important problems of a practical nature, entirely aside from the

observation of characteristics or their symbolic designation, e.g. by words. To indicate what kind of problems arise from the mere act of grouping, let us consider a situation in which we may assume—somewhat arbitrarily—that no problems arise in the observation of significant characteristics and their consistent and unambiguous designation by appropriate symbols. Such a system might be represented by a number of small objects, each of which has one characteristic from 10 sets, such as might apply to blocks for children. They might include shape, material, color, texture, largest transverse dimension, arabic numerals, roman numerals, lower case letters, upper case lettering and special marking, as listed below.

1) Shape—10 characteristics (cube, other parallelepiped, sphere, tetrahedron, pyramid, truncated pyramid, cone, truncated cone, cylinder, ellipsoid).

2) Material—10 general characteristics, with 10 specific characteristics under each (pure metal, ferrous alloy, non-ferrous alloy, plastic derived from cellulose, other plastic, glass, other ceramic, wood, edible material, other material).

3) Color—10 general characteristics, with 10 specific characteristics under each (red, yellow, green, blue, orange, purple, gray, brown, pink, cream-buff).

4) Surface texture—10 characteristics (painted, rough, smooth, polished, various forms of surface protuberance or indentation).

5) Largest transverse dimension—10 ranges with 10 specific values for each.

6) Arabic numerals—0–9 (total of 10 characteristics)

7) Roman numerals—I–X (total of 10 characteristics)

8) Lower case letters—a–z (total of 26 characteristics)

9) Upper case letters—A–Z (total of 26 characteristics)

10) Special marking—10 characteristics, &, $, ¢, ℔, @, #, /, ;, ?, :.

If each object in our collection is characterized by one characteristic from each of the ten sets and if, furthermore, our collection contains an object for each of such combination of characteristics, then we would have $10 \times 100 \times 100 \times 10 \times 100 \times 10 \times 10 \times 26 \times 26 \times 10 = 6.76 \times 10^{13}$ objects in our collection.

It is, perhaps, immediately obvious that the objects in our collection might be classified into groups in the same way as documents may be grouped into fixed arrays, e.g. pigeonholed classification of patents. It is instructive to consider the various ways in which such classification systems might be set up. For example, we might select either shape or material, or color and shades of color. Having made this first selection for the main subdivision, any one of the 9 remaining sets of characteristics might be chosen in setting up the second subdivision.

The number of distinct classification systems that may be set up on 10 sets of characteristics may be determined by multiplying $10 \times 9 \times 8 \times 7 \times 6 \times 5 \times 4 \times 3 \times 2 \times 1$, a product which mathematicians denote as 10!

In general, if L is the number on sets of characteristics, then the number, U_c, of classification systems that may be set up as indicated above will be given by the equation $U_c = L!$. Obviously L! increases very rapidly as L becomes larger. The following examples illustrate this point:

L	U_c	L	U_c
1	1	6	720
2	2	7	5,040
3	6	8	40,320
4	24	9	362,880
5	120	10	3,628,800

If we now direct attention to the mathematical model as specified by the described criteria, it may be observed that establishment of classification systems may be accomplished by basing subdivision not on the individual characteristics as listed but on a less discriminating characterization. Thus we might decide, with regard to shape, not to distinguish between spheres and cubes or between cones and pyramids or between truncated pyramids and truncated cones or between cylinders and ellipsoids. Such a decision would then reduce to 6 the number of shape characteristics. This illustrates one way in which basic characteristics may be combined to generate additional characteristics which we will speak of as 'derived characteristics.' More specifically, these examples illustrate how basic characteristics may be combined on an 'and/or' basis to generate derived characteristics. Such and/or combinations we shall refer to as derived characteristics based on logical sums. In the general case, a logical sum of basic characteristics is not limited to two characteristics as in the example just cited. We might generate a derived characteristic as the logical sum of: cube and/or sphere and/or pyramid and/or cone, which we might symbolize as: A + B + C + D.

Derived characteristics may be generated also by basing their definition on the simultaneous presence of two or more characteristics, each from a different set. Thus we might define as a derived characteristic, the combination of properties 'spherical shape, made of gold, painted red.' Since this method of generating derived characteristics requires that each of two or more basic characteristics shall be present, we may, in general, denote such derived characteristics by a logical product, which may be represented, $A \cdot B \cdot C$.

It is perhaps obvious that derived characteristics may also be defined in terms of more complex combinations of basic characteristics. Thus we might have the derived characteristic 'sphere (A) or cube (B) made of gold (C) without the Roman numeral IV (D).' Such a definition may be represented symbolically by $(A + B)(C - D)$.

It will be recalled that the classification systems that we have been considering are built up of subdivisions, each of which is defined in terms of a combination of characteristics, with each combination containing no more than one characteristic from any one of the sets into which the characteristics are organized. In somewhat more formal language, all the subdivisions of such classification systems are built up by establishing logical products of characteristics taken from sets of mutually exclusive characteristics. It is perhaps obvious that such classification systems constitute a special type. The more general types of system would include subdivisions defined not only in terms of logical products but also logical sums and logical differences. Hence, our mathematical analysis of our model system must be regarded as capable of very considerable extension.

Classification and Definition of Concepts and Terminology. Our mathematical model also provides a basis for considering various relationships between *1*) definition of classes and subclasses in a classification system and *2*) the definition of concepts and of terminology used to express and record concepts. These relationships are perhaps most obvious in the field of taxonomy. For example, a fish is an animal having a certain set of characteristics. The same is true not only of the full range of terminology used in taxomony, such as coelenterate, paramecium, etc., but also of such everyday concepts and words as tree, house, chair, shirt, etc. With everyday words the sharpness of definition may be less than with terminology subject to scientific control. Such lack of precision in the definition of many concepts and words is, of course, a factor that requires consideration when conducting such operations as indexing, classifying and coding information for machine searching. Even when a tolerable level of precision in definition is attained, we observe that words vary in scope of meaning. For example, the words *animal*, *mammal*, *dog*, *fish*, *bird* and *tree* all refer to living organisms, but to different sets or groups of the same. Further, we observe that there is a tendency for words to overlap in meaning (more or less near synonyms). These factors must be taken into account in constructing classification systems, indexes, codes for machine searching and similar systems for analyzing information and facilitating its retrieval, correlation and utilization. These ideas have been reviewed in detail elsewhere (1).

Practical Problems in Classification. In our previously described mathematical model, we have pointed to certain problems that arise from the very act of establishing fixed groupings of objects whose characteristics are well-defined and precisely observed. This mathematical model emphasizes the point that a very large number of alternate possible groupings may be established on the basis of a relatively small number of sets of observational characteristics. This means, in practical terms, that the possible usefulness of various alternate groupings must be accorded due consideration. In some circumstances, it may be true that some one grouping is obviously much more useful than other possible groupings. This might be the case, for example, when grouping tableware and kitchen utensils in a cupboard. It cannot be assumed, however, that there exists some grouping which is equally useful or effective for all purposes, particularly with deepening understanding resulting from scientific progress as discussed below. Apparently there has been confusion between the general act of grouping, on the one hand, and the limitations of providing groupings when using such devices as pigeonholes or similar compartmentalizing devices. In the extreme case, the limitations of fixed compartmentalization appear to be used as arguments to support the contention that some one set of fixed grouping can and must be established to meet diverse purposes. Consideration of the mathematical model, it is hoped, may aid in overcoming this ancient fallacy.

Our mathematical model was set up in such a way that we might reasonably assume, for purposes of analyzing certain important consequences of the act of grouping, that no problems would arise in the observation of significant char-

acteristics and their designation by appropriate symbols. Any consideration of the fundamental problems of classification cannot, however, avoid mention of those problems which relate to detection, observation, measuring and recording of characteristics. There is a close parallel here between underlying problems of characterization and of research planning. In classifying, decisions must be made as to which characteristics are to serve as the basis for classification. In research planning we must decide what to observe, and what to measure. Closely related questions are "How reliable are our observations?" "How accurate are our measurements?" In terms of our mathematical model, there might be uncertainty in deciding whether a given object is to be regarded as a cube or some other parallelepiped or possibly a truncated pyramid. In fact, a classificatory decision as to which characteristic is to be attributed to a given object might well depend on the precision with which certain measurements are performed. Similarly, the characteristics of a given substance may remain unsuspected for centuries. Thus, the Indians of the Colorado plateau observed that the mineral carnotite has certain color characteristics which they exploited for artistic purposes. Their observations were insufficient for them to imagine how this same mineral could be used to construct weapons infinitely more powerful than their sharpest arrows. The characteristics of this mineral that make it the key material in producing atomic bombs remained unexplored by these primitive tribes. Similarly, the scientific discoveries of the present era, which appear highly sophisticated to us, may seem primitive, perhaps even naive, to our descendants. Their more profound appreciation of the importance of characteristics overlooked by us today may provide the basis for a completely different system for classifying not only minerals and chemical elements but also plants, animals and other organisms.

Once currently available knowledge has provided as effective a basis as possible for conducting observations and determining characteristics to serve as a basis for classification, another problem realm is entered. This concerns the designation of characteristics in terms of appropriate symbolism. Here one has not only the problem of nomenclature to designate individual characteristics, but also the closely related problem of terminology to designate groups of objects which are characterized by certain sets of characteristics. Discussion of our mathematical model pointed out how an individual characteristic of the objects in question might be designated by word, e.g. *sphere*. Further, the model showed how we might establish terminology to refer to any given combination of characteristics. Such terminology might then become the name of a class of objects having a given set of characteristics in common.

It should be emphasized again that admission or denial of membership by a given entity to a given class depends not only on those combinations of characteristics that define the class but also on the definition of the characteristics themselves, as well as on the accuracy with which such characteristics are determined when investigating a given entity.

These problems of establishing important characteristics, of detecting them when studying individual entities and of deciding which combinations of characteristics define a useful grouping, are quite independent of the means that may be used for actually establishing such groupings, or at least preparing listings which symbolically represent a useful and desirable grouping. The classical approach to these problems has been to assume—either on the basis of intuitive conviction or wishful thinking or possibly both—that certain characteristics are 'essential,' that they represent 'essences' and that other characteristics are 'non-essential' or 'accidents.' To the Indians using carnotite in their artistic endeavors the color of the mineral carnotite was doubtless essential, whereas the atomic weight and nuclear properties of uranium would be for them not only accidental properties but even properties that remained undetected and unsuspected. On the other hand, to the present day physicist, who is interested in the release of nuclear energy, the color of the mineral carnotite may well be an accident, whereas the essential properties are those inherent in the atomic nuclei as we understand such entities at the present stage of development of science. Attempts to distinguish between essential and accidental characteristics depend in the last analysis on judgments as to which characteristics are important. These judgments in turn will be determined by the status of knowledge at some given time. To confuse such judgments with logical conclusions may well lead to grotesque situations in which it will be observed that the word 'logical' is being used as a synonym for 'useful.'

This confusion of 'logical' with 'useful' is likely to appear in particularly striking form when the

device used for achieving classification is such that only one (or scarcely more than one) set of permutations of characteristics may be set up for various practical reasons. Thus, when objects are to be grouped in pigeonholes or similarly listed in a fixed printed array, the impossibility of a large number of alternate groupings or of multiple listings compels, perforce, that some one permutation of characteristic be accepted as standard. It may well be that a standard listing of this type, or a corresponding standard grouping of actual physical objects, may be highly useful. Everyday experience provides plenty of examples to illustrate this point. The homely example of kitchenware in a housewife's cupboards has already been referred to in this connection. Such a classification greatly facilitates the preparation and serving of food. Other equally valuable purposes are served by such fixed configurations of characteristics as the taxonomical systems of a biologist. The usefulness of these systems is not a matter of abstract logic but rather of judgment tempered by experience as to which groupings are, in fact, most useful in dealing with practical situations or in conducting research to develop new knowledge. Furthermore, the fact that a certain fixed array is useful for one or several important purposes provides no guarantee that the grouping will serve all purposes in an acceptable fashion.

As is well known—and is illustrated by our mathematical model—in dealing with entities, e.g. biological organisms having a multiplicity of characteristics, a wide range of permutation of characteristics can readily be set up. As our mathematical model has shown, the number of such possible permutations becomes extremely large even with a relatively small number of sets of characteristics. In practical situations, it may appear possible to reject the overwhelming majority of the alternate possible permutations of characteristics as devoid of utility. Such rejection, as discussed above, is a matter of experience and judgment based on the present status of scientific development. Even when such rejection appears fully justified, it is a matter of common observation that there are often at least several alternate possible permutations between which choice is difficult. Decisions as to selection of the 'standard' or 'official' classification can then be based at best on utility under special circumstances. The conflicting viewpoints of specialists working in different areas or approaching the same problems from different points of view may make it virtually impossible to choose among alternate combinations of characteristics in establishing classification systems. Under such circumstances, it is unavoidable—human nature being what it is—that misunderstandings and conflict should arise. Such misunderstandings become more difficult to clear up and the accompanying conflicts may become bitter, if it be assumed that a universal classification equally effective for all purposes is a realistic goal. Such an assumption is, of course, completely unwarranted, and it was to make this point as clear as possible that our mathematical model was presented in some detail.

Newer Devices for Accomplishing Classification. The limitations of hierarchical classification, that is to say, the limitations of working within the framework of only one permutation of a given set of characteristics, have provided motivation, during recent years, for the development of methods which permit such limitations to be transcended. It would lead us too far to attempt to describe these new methods in detail (2–4). Suffice it to say, for present purposes, that the new methods permit individual characteristics to be independently registered with the aid of one means or another. A particularly simple device for this purpose has been found in hand-sorted punched cards. With these cards, each characteristic may be recorded as a notch at the periphery of the card and these notches may be used, either singly or in combination, to select cards that contain information relating to some one characteristic or to various possible combinations of characteristics. The important point here is that a predetermined set of characteristics is not the basis for establishing a grouping to serve some purpose. Rather, analysis of the purpose leads to formulating a corresponding set of characteristics, and these, in turn, provide the basis for conducting selecting operations and for establishing the desired grouping.

Even with such simple mechanical devices as hand-sorted punched cards, selecting and grouping operations may be formulated in highly diverse fashion. To start with a simple case, hand-sorted punched cards permit selections to be made on the basis of logical products. In other words, cards punched to designate a set of characteristics may be selected on the basis of all of certain characteristics being required. Such a requirement, for example, may be symbolized by $A \cdot B \cdot C \cdot D$. Alternately, cards may be selected if punched to indicate any one of several characteristics. Such a search requirement, which cor-

responds to a logical sum, may be symbolized, for example, by A + B + C + D. Selection may also be based on the requirement that some characteristic shall be present but another must be absent. This requirement corresponds to a logical difference, which may be symbolized, for example, by A − B. Similarly, certain search requirements may be formulated as more complex logical relationships and the corresponding selecting operations may be performed with the aid of hand-sorted punched cards. For example, a search based on a combination of characteristics symbolized as (A + B) (C + D) − E may be carried out.

Hand-sorted punched cards are, of course, a particularly simple device. It is perhaps worth while to add that the notational representation of logical product, sum, difference and complex relationships, as mentioned in the preceding paragraph, has provided the basis for constructing automatic equipment whose modus operandi and capabilities might be summarized as follows (5, 6).

1) Record sequences of symbols by punching paper tape. In this way the characteristics of documents may be recorded one after another for subsequent search by the selector. (Individual symbols and combinations of symbols may be used to record the characteristics of documents in the same way that individual letters and combinations of letters are used to denote words and punctuation in ordinary writing. It should also be noted that meaning may be ascribed to any combination of symbols as may be appropriate.)

2) Read the punched paper tape and convert the patterns of holes used to record successive symbols into corresponding pulses of electronic pulses, which then activate the discriminating unit.

3) Detect those characteristics and combinations of characteristics which typify the subject contents of documents that are of pertinent interest. (The discriminating unit is conditioned to detect such characteristics by appropriate wiring of a plug board prior to initiating a given search.)

4) Type out automatically the serial numbers of those documents whose characteristics correspond to the requirements of a given search. (The scope of a search will be expressed by specifying that the documents of pertinent interest shall have some one characteristic or some combination of characteristics. Possibilities for specifying combinations of characteristics are outlined below.)

The sequences of symbols may be organized into combinations analogous to 'syllables' from which 'words' may then be built up and from which, in turn, combinations analogous to 'phrases', 'sentences' and 'paragraphs' may be built up. If the capital letters A, B, C, D, etc. are used to designate individual symbols, then letters with subscripts may be used to designate various levels of combinations, as follows:

A_1, B_1, C_1, D_1, etc. for *syllables*
A_2, B_2, C_2, D_2, etc. for *words*
A_3, B_3, C_3, D_3, etc. for *phrases*
A_4, B_4, C_4, D_4, etc. for *sentences*
A_5, B_5, C_5, D_5, etc. for *paragraphs*
A_6, B_6, C_6, D_6, etc. for *messages*

This ability to organize characteristics into sets analogous to 'phrases,' 'sentences,' etc., is important in preventing false association of characteristics when searching. For example, by proper 'phrasing' it is possible to prevent the properties of one chemical compound being incorrectly attributed to some other compound. Detailed rules for such use of 'phrases,' 'sentences,' 'paragraphs,' etc., in encoding of abstracts will be presented in a book now in press (7).

At any level, which we may term the n^{th} level, each combination will consist, in general, of a number of component combinations at the $n - 1$ level. Each of several n^{th} level combinations, denoted by A_n, B_n, C_n, D_n, etc. may be specified in terms of component units designated by A_{n-1}, B_{n-1}, C_{n-1}, D_{n-1}, etc. Thus, in conducting a search, it may be specified, as a condition that a document will be identified as being of pertinent interest, that at least one n^{th} level combination shall be characterized by certain component units. Specification of the component units may be set up on the basis of the following relationships. It may be specified that—

1) All of several components units must be present. This requirement constitutes a logical product that may be symbolized, for example, by:

$$A_{n-1} \cdot B_{n-1} \cdot C_{n-1}, \text{ etc.}$$

In specifying logical products, further requirements as to order may be imposed. Thus, for example, it may be required that all components specified by a logical product shall occur in sequence. For example, it may be required that A_{n-1} shall be followed by B_{n-1} and it in turn by

C_{n-1}. This requirement may be symbolized by:

$$<A_{n-1} \cdot B_{n-1} \cdot C_{n-1}>$$

Other sequential order, e.g. the reverse, might also be specified as denoted by:

$$<C_{n-1} \cdot B_{n-1} \cdot A_{n-1}>$$

2) Any one of several component units or, alternately, one or more of several component units must be present. This requirement constitutes a logical product that may be symbolized, for example, by:

$$A_{n-1} + B_{n-1} + C_{n-1}, \text{ etc.}$$

3) At least one component unit must be present but at least one other component unit must be absent. This requirement constitutes a logical difference that may be symbolized, for example, by:

$$A_{n-1} - B_{n-1}$$

Here, also, order may be designated. Thus it may be specified that B_{n-1} may not follow A_{n-1}. This requirement would be symbolized by:

$$<A_{n-1} - B_{n-1}>$$

Alternately, it might be specified that B_{n-1} may not precede A_{n-1} and this would be symbolized by:

$$<-B_{n-1} \cdot A_{n-1}>$$

4) Combinations of component units expressed by complex logical relationships must be present. Such logical relationships as the following may be specified:

$$(A_{n-1} \cdot B_{n-1} - C_{n-1})\ (D_{n-1} + E_{n-1})$$

$$(A_{n-1} + B_{n-1})\ (C_{n-1} - D_{n-1})\ E_{n-1}$$

$$(A_{n-1} - B_{n-1})\ (C_{n-1} \cdot D_{n-1} - E_{n-1})$$

Any such complex logical relationship may be set up as required at any level. Such complex logical relationships may also involve specification of sequential order. Using the symbols $< >$ to denote order as before, we might specify such search requirements as:

$$(<A_{n-1} \cdot B_{n-1} - C_{n-1}>)\ (D_{n-1} + E_{n-1})$$

$$(A_{n-1} + B_{n-1}) < (<C_{n-1} - D_{n-1}>)\ E_{n-1}>$$

$$<(<A_{n-1} - B_{n-1}>)\ (C_{n-1}\ D_{n-1} - E_{n-1})>$$

Application of these capabilities means that syllables' may be specified in terms of component symbols, e.g. letters; 'words' may be specified in terms of 'syllables'; 'phrases' in terms of 'words'; 'sentences' in terms of 'phrases'; 'paragraphs' in terms of 'sentences' and 'messages' in terms of 'paragraphs.'

Prior to conducting a searching operation, the machine must be conditioned so that combinations of characteristics corresponding to the search requirements will be automatically detected when the machine reads the tape in which successive sets of characteristics have been recorded. Each set of recorded characteristics will correspond, of course, to a single document. The conditioning of the machine is accomplished by wiring a plug board. As already noted, detection of the fact that the characteristics of a certain document fulfill the search requirements is recorded by automatic typing of the document's serial number. If pieces of photographic film were used *a*) to record characteristics, in coded form, as patterns of transparent spots on an opaque background and *b*) to record, in microphotographic form, the document in question, then the detection of fulfillment of search requirements could be made to select the corresponding piece of photographic film. Thus the identification of pertinent documents can be more closely linked with their delivery for perusal and use (8, 9).

At present the above specified equipment (3) has been designed and constructed to conduct ten searches simultaneously. Each search is assigned a different number, which is automatically typed immediately following the serial number of each document whose characteristics are found to correspond to the search requirement in question. If a given document fulfills the requirements of several simultaneously conducted searches, each of the latters' identifying numbers are typed immediately following the serial number of the document in question.

Simultaneously conducted searches may be directed to completely unrelated information requirements. For example, one search might be directed to documents on production of lithium ore in Canada during the year 1955 and a simultaneously conducted search might be directed to the use of manganese containing alloys for treating cast iron to improve its properties. Simultaneously conducted searches may also be directed to interrelated search requirements. For example, the following interrelated searches could be conducted simultaneously and documents pertinent to each could be identified: *1*) Application of the Photovolt Multiplier Photometer to absorption

spectrophotometry; *2*) Application of electronic light measuring devices to absorption spectrophotometry; *3*) Use of electronic devices in absorption spectrophotometry; *4*) Use of electronic devices in spectrophotometry; *5*) Instrumentation in spectrophotometry.

Such possibilities of conducting interrelated searches are particularly important in correlating information scattered among different documents.

Coordination of Classificatory Procedures. In closing, it should be emphasized that the capabilities in establishing the groupings provided by such electronic equipment enables virtually any permutation or combination of characteristics to be used as the basis for effecting a grouping. Providing this capability does not, of course, exclude the possibility of establishing a fixed configurational arrangement as provided by classification systems of traditional design. In many situations it seems likely that the newer methods, based on the ability to effect groupings as required, and the traditional methods of predetermined arrangement by hierarchial classification, may provide supplementary advantages and capabilities. The two methods may thus serve to complement each other and to facilitate the utilization and development of knowledge in a much more effective way than would be possible with either used alone. It seems likely that this may, indeed, be the situation in the biological sciences.

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AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

Symposium on Molecular Heterogeneity of Hemoglobin[1]

Chairman: DAVID L. DRABKIN

INTRODUCTION: HEREDITY AND ENVIRONMENT IN STRUCTURE OF HEMOGLOBIN[2]

DAVID L. DRABKIN

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NINE years have elapsed since our Society's previous Federation symposium on Hemoglobin (1). Happily, at the present session my participation is more strictly confined to that of timekeeper and second man outside-of-the-ring, who introduces the principals.

Other important advances in this area were covered 2 years ago at a Ciba Foundation Symposium, under the able chairmanship of Claude Rimington (2). These embraced the elucidation of the pathway of biosynthesis of the hemin of hemoglobin, the beginnings of knowledge of the rôle of enzymes in this biosynthetic process, the production of an experimental state of porphyria, and, especially noteworthy, the establishment of the structure of porphobilinogen by Cookson and Rimington (3) and the demonstration that this substituted monopyrrole is most probably the immediate metabolic precursor of porphyrins (3; cf. also ref. 2). Moreover, it now appears clear, as originally proposed independently by Theorell and his colleagues (4) and by the writer (5, 6), that the biosynthetic capability for hemin chromoproteins is possessed inherently by all aerobic cells, and not limited to a particular tissue or body organ. Mention is deserving also of a recent clarification of one aspect of the degradative metabolism of hemoglobin. A satisfactory explanation of the direct and indirect van den Bergh reactions presumably has at last been made, the indirect being accounted for by free bilirubin, the direct by the conjugated bilirubin glucuronide (7, 8).

This conference, accordingly, is restricted largely to those developments, their meaning and consequences, in which human hemoglobin, long regarded as a single entity, has been disclosed to be a multiple substance, with new recruits, normal as well as abnormal, ever joining the line of marchers already grown to a fairly large (if not confusing) number. F. J. W. Roughton, who has remained admirably steadfast in his careful, systematic analysis of the oxygenation equilibria of hemoglobin (9), once remarked that the blood pigment was a lovely but demanding mistress. I am afraid that he and other pursuers of this lady —a mysteriously dimpled lady, according to Max Perutz who has his own artful approach—must now gird themselves for dealing with a harem of perhaps disconcertingly different personalities. Without burdening this metaphor, think only of the labor required to repeat Roughton's type of study on ten different human hemoglobins, although the needfulness of such an effort may be questioned.

In this presently expanding field, a look backwards is desirable, and table 1 is devoted to a résumé of 'landmarks' in the first 100 years of the history of hemoglobin. From the names in this table it is especially appropriate on this occasion to single out and memorialize a distinguished Chicago physician, James Bryan Herrick (1861–1954). On December 26, 1904 (the published report appeared 6 years later, ref. 37), he made the original observation of a "freakish poikilocytosis" in the blood of a severely anemic

[1] Chicago, April 16, 1957.

[2] Much of the more recent work in the writer's laboratory on chromoproteins has been aided by grants supplied by the Office of Naval Research and the Bureau of Medicine and Surgery, Department of the Navy, and was done under contract between the Office of Naval Research and the University of Pennsylvania.

TABLE 1. LANDMARKS IN FIRST 100 YEARS OF HISTORY OF HEMOGLOBIN

Year	Investigator	Report
1827; 1831	Lecanu	Blood (hemoglobin) contains iron (10)
1832	Blaud	Antichlorotic pill of ferrous iron (11)
1840	Hünefeld	Crystallization of hemoglobin (12)
1844–1851	Mülder	Iron split from 'hematin' (13)
1850	von Kölliker	Original claim that myoglobin is distinct from hemoglobin (14)
1852	Teichmann	Teichmann's crystals of chlorohemin (15)
1859	Kühne	Injected hemoglobin changed to bile pigment; large injections produce hemoglobinuria (16)
1864	Stokes	Spectral change on deoxygenation of oxyhemoglobin (17)
1865	Harley	Paroxysmal hemoglobinuria described (18)
1866[a]	Körber	Hemoglobins of different species are different, since they are degraded at different rates by means of alkali or acid (19)
1867	Thudichum	Hematoporphyrin ('cruentine') produced from hemoglobin (20)
1867	Lankaster	Discovery of chlorocruorin (21)
1868	Zuntz	Red cells (hemoglobin) necessary for increase in bicarbonate upon addition of CO_2 to blood (22)
1868	Biermer	Description of pernicious anemia or Addison-Biermer anemia (23)
1873; 1876	Vierordt	First spectrophotometric measurements on hemoglobin (24)
1876	Cazeneuve	Iron content and elementary composition of chlorohemin (25)
1882	Banti	Description of splenic anemia attended by biliary cirrhosis (26)
1886	MacMunn	Discovery of myohematins and histohematins (cytochromes) (27). Appreciation of MacMunn's work delayed for many years due to criticism by Hoppe-Seyler (28)
1886	Zinoffsky	First accurate analysis of iron content of hemoglobin (29)
1888[a]	von Krüger	Revival of Körber's work on rates of denaturation of different hemoglobins with alkali—the so-called von Krüger reaction (30)[b]
1889	von Jaksch	Description of pseudoleukemic anemia of infants (31)
1891	Viault	Polycythemia associated with altitude anoxia (32)
1893[a]	Bertin-Sans and Moitessier	Early suggestion that hemochromogens are compounds of hemin and a nitrogenous base (33)
1903	Bohr	Early accurate oxygen dissociation curve of hemoglobin and effect of CO_2 (34)
1909[a]	Reichert and Brown	Exhaustive treatise on crystalline hemoglobins; α and β crystalline types frequently found (35)
1909–10	Barcroft and Hill	Interpretation of S-shaped oxygen dissociation curve (36)[c]
1910[a]	Herrick	Discovery of sickle cell anemia; original observation made in 1904 (37)
1912	Peters	Establishment of relationship between oxygen and iron in oxyhemoglobin (38)
1914	Christiansen, Douglas and Haldane	At same CO_2 tension, more bicarbonate found in deoxygenated than in oxygenated blood (39)
1916	Hasselbalch	Deduction that HbO_2 is a stronger acid than Hb; explanation of observation of Christiansen, Douglas and Haldane (40); cf. also Henderson (41)
1925	Adair	Deduction from osmotic measurements that hemoglobin has a molecular weight of order of 70,000 and is a four Fe atom structure (42)
1926	Svedberg and Fahraeus	Adair's view confirmed by sedimentation rate in ultracentrifuge (43)
1926	Van Slyke	Classical monograph on hemoglobin and the regulation of neutrality in the organism (44)
1927[a]	von Krüger and Bischoff	Differentiation of fetal and adult human hemoglobin through alkali resistance of the former (45)
1927[a]	Cooley	Description of erythroblastic or Mediterranean anemia (thalassemia) (46)
1928	Henderson	Classical monograph on blood as a physicochemical system (47)
1928–29	Henriques	Discovery of carbamino hemoglobin (48)
1930[a]	Haurowitz	Deduction from 'biphasic' alkali resistance curve that hemoglobin of newborn infant is heterogeneous, about 80% fetal and 20% adult (49)[d]

TABLE 1—CONTINUED

Year	Investigator	Report
1930[a]	Schenck	Claim that hemoglobin in Pernicious Anemia is, like fetal, more resistant to alkali and hence different (50)[e]
1931[a]	Geiger	Earliest definite evidence (cataphoretic) that normal adult hemoglobin of dog, sheep, ox, ass and man is heterogeneous (51)
1933[a]	R. oowitz	Claim that hemoglobins of man and animals at altitude differ cataphoretically from those at sea level (52)

[a] Especially pertinent to present considerations. [b] That the Körber-von Krüger reaction was essentially the change of hemoglobin to hemochromogen and that the rate could be followed quantitatively by spectrophotometry was shown by Drabkin and Austin (53). [c] A memorial symposium in honor of Sir Joseph Barcroft, the proceedings of which have been published in a book, *Haemoglobin* (54), was held at the University of Cambridge in June 1948. [d] 'Biphasic' alkali resistance curves obtained by Drabkin on solutions of normal adult hemoglobin (55). [e] Not confirmed (cf. 56), but Schenck's suggestion of the possibility of abnormal hemoglobins is of historical interest.

young male negro. This was the modest, but essential first step—the discovery by a careful and astute observer of a new disease entity, sickle cell anemia. Some years after the publication of Herrick's findings, evidence that this disease was inherited began to appear (57), but the nature of the genetic involvement was clearly defined only many years later by Neel (58).

Figure 1 outlines a sequence of events, which may be representative of a pattern in the development of scientific information, and is based on Linus Pauling's own account in his Harvey Lecture (59). In this scheme, it is pertinent to emphasize the point that *disease is the great teacher*. The classical paper of Pauling, Itano, Singer and Wells, *Sickle cell anemia, a molecular disease*, appeared in 1949 (60). I believe that the impact of this important contribution lay not only in the disclosure of the investigative findings, but in the words 'molecular disease' in the cogently chosen title. This report upon the first of an enlarging group of molecular diseases came less than a year after our earlier symposium on hemin pigments and chromoproteins. There is every reason to believe that much of the work of Pauling and his associates (59, 60) on the hemoglobin of sickle cell anemia had already been done in 1948, making that Federation conference somewhat out-of-date at the time it was in session (1).

In figure 2 a scheme of the biogenesis of the red cell is presented. It avoids such terms as 'alleles' and 'homozygous,' with which the hemoglobin chemist is now forced to contend, and may serve as a deceptively easy introduction to the genetic complexities of the problem, which no doubt will be touched upon by Dr. Itano and dealt with in detail by Dr. Zuelzer. To start with, it should not be forgotten that the red cell and its hemoglobin or hemoglobins have finite life spans.

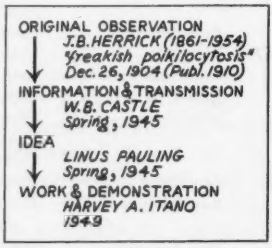


FIG. 1. Disease challenges, and supplies the problems—a pattern of progress.

Consequently, each drawn sample of blood must contain some red cells undergoing obsolescence, and such red cells may in a sense be regarded as 'morbid' (cf. 61). What about the hemoglobin they contain? Furthermore, it has not been settled whether or not the red blood cells of adult man are a single-lived species. There may be cells which have a short, others which have a longer life span (62–64). Within the framework of these original uncertainties, at least four types of genetic influences appear to have been disclosed in regard to hemoglobin and red cell biogenesis (see legend to fig. 2).

In figure 3 (A), Roughton's lovely mistress is presented, together with sickle cell hemoglobin, (S), homozygous C hemoglobin, (C), and a newcomer, hemoglobin X, of attractive and beguilingly different appearance, who has crept into the harem. All of these hemoglobins have been crystallized by Drabkin's method (65), now commonly employed in the study of molecularly heterogeneous hemoglobins, and the tetragonal, bipyramidal crystals (A, S, and C) have been called 'Drabkin crystals,' while the concentrated hemolysate from which they precipitate has been referred to as 'Drabkin hemolysate.' It is most flattering to me to have them so labeled, but I

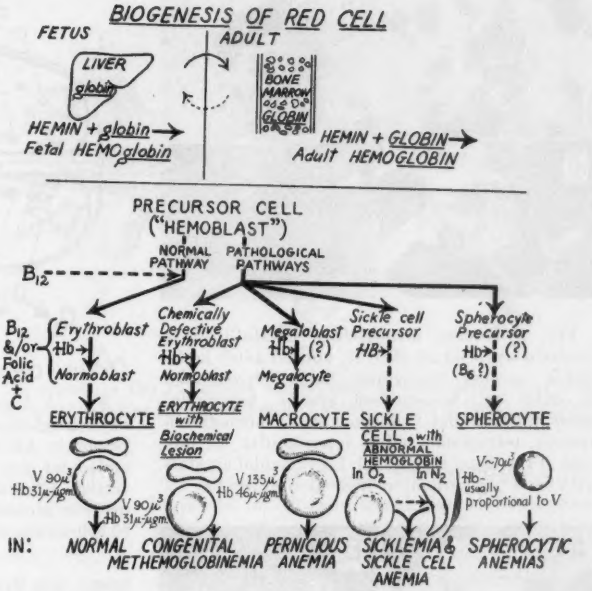


FIG. 2. *Upper:* A concept of biogenesis of fetal and adult hemoglobins, which have identical hemins but different globins; *solid arrow* suggests the shift from fetal to adult hemoglobin production, which actually is initiated in the last months of gestation (49), *broken arrow* may suggest either a reversion to or retention of an embryonal pattern. The liver (shown) is not the sole organ involved in so-called extramedullary blood production. *Lower:* A scheme of red cell biogenesis, consonant with known facts (61). As contrasted with the older view still current in many texts on hematology, normal and abnormal erythrocytes are pictured as having independent origins. Inherited biochemical lesions of at least four types may be postulated. These are represented by *a*) lack of enzymic equipment for the reversion of ferrihemoglobin (cf. refs. 1, 61), as in *congenital* or *familial methemoglobinemia* (red cell abnormal; hemoglobin normal); *b*) a genetically determined alteration in the biosynthesis of hemoglobin (58), as in *sickle cell anemia* (red cell abnormal (?); hemoglobin abnormal); *c*) a genetic inhibition of normal adult hemoglobin synthesis and retention of embryonal synthetic pattern (46, 59), as in the spherocytic *familial erythroblastic* or *Mediterranean anemia* (red cell abnormal; hemoglobin fetal type) and *d*) a genetic absence of the *intrinsic factor* (in stomach mucosa), thereby making B_{12} unavailable to the bone marrow and favoring the production of the 'morbid' megaloblast in place of the erythroblast, as in classical *pernicious anemia* (red cell abnormal; hemoglobin may be normal, refs. 50, 56). Other forms of megaloblastic anemia, which are not genetically determined, are recognized.

may point out that claims of the crystallization of human hemoglobin have been made for over 100 years (12) and my effective crystallization of human hemoglobin and discovery of the tetragonal configuration had the benefit of earlier work, particularly that of Cannan and Redish (66), Morgan (67) and Green, Cohn and Blanchard (68).

In figure 4 a better view of the crystals, designated hemoglobin X, is shown. On this occasion it is most pleasant to announce presumptive evidence—at this stage largely dependent on the almost equally hazardous bases of crystallographic configuration and intuition—of yet another adult human hemoglobin. It came first to my attention in June, 1955; indeed, I looked for it. It is from a female subject with 'chronic' extramedullary blood production (a patient followed for many years by Dr. Maxwell Scarf of Philadelphia). In an extensive experience with the crystallization of human hemoglobins, this is the only instance in which orthorhombic crystals (legend, fig. 4) have been obtained under the conditions of Drabkin's crystallization technique. It is evident that the specimen contains both the usual 'Drabkin crystal' and the 'new' orthorhombic variety. The adage "beauty is but skin deep" appears to apply to hemoglobin crystals. In the case of S and C, the tetragonal bipyramids are the same as those of A, but this structure masks subtle internal differences. In the case of X the outward form is different than A, but there the difference, with our present probes, essentially ends. It should be stated that X is presumably not fetal hemoglobin. It is a curious hemoglobin —not a pathological one, nor yet normal. It exists in that shadowy borderland between the two, and may be said to arise in response to the body's desperate need of an oxygen carrier when the bone marrow fails to make enough. Perhaps hemoglobin X belongs to a new class, an adap-

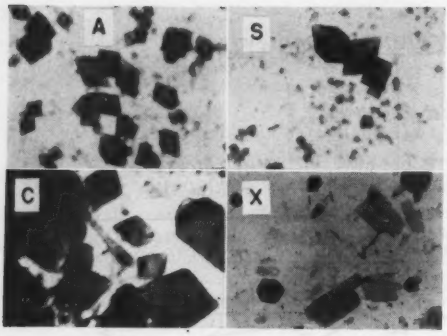


FIG. 3. Human hemoglobins crystallized by Drabkin's procedure (65). *A*, normal adult hemoglobin: system, tetragonal; habit, bipyramidal. *S*, sickle cell hemoglobin: system, tetragonal; habit, bipyramidal. *C*, homozygous C hemoglobin: system, tetragonal; habit, bipyramidal. *X*, mixture of the usual tetragonal, bipyramidal crystals, with 'new' orthorhombic hemoglobin (see fig. 4).

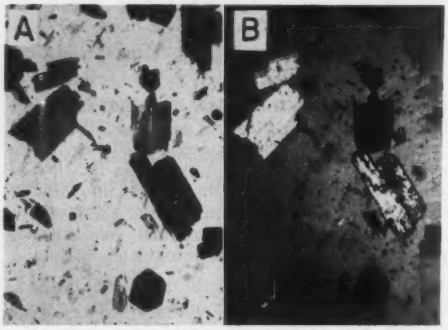
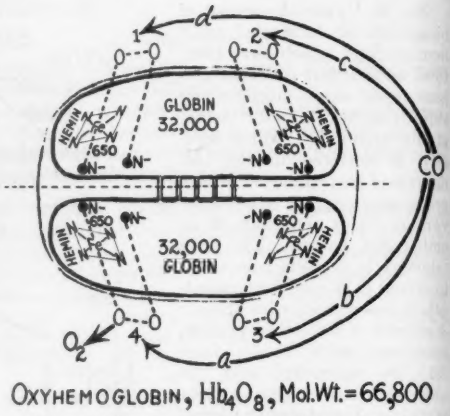


FIG. 4. Hemoglobin X, crystallized by Drabkin's procedure (65) from blood of patient with 'chronic' extramedullary blood production. The same microscopic field is shown, with polarization optics uncrossed (*A*) and crossed (*B*). The habit of the unusual crystals (designated X) is thin, lath-like prisms with growth on long axis, forming tabular plates, which are highly pleochromatic and birefringent. The horizontally oriented crystals are parallel with a 90° axis, rendering obvious the straight extinction. The sign of elongation was negative, and the biaxial brush pattern was obtained on conoscopic observation. Hence, the symmetry is orthorhombic, in contrast with the usual tetragonal.

tive hemoglobin, in the same sense that cytochrome *c* is adaptive (64 and 69).

Environment. Besides the abnormal hemoglobins, we must now also consider the presumptive existence of two or more normal adult hemoglobins. My personal position in this area, I am glad



OXYHEMOGLOBIN, Hb_4O_8, Mol.Wt.=66,800

FIG. 5. Representation of the molecule of oxyhemoglobin, drawn in 1945, which still fits presently known facts. The concept of partial molecular poisoning with CO (61) is also illustrated. A minor change is that it is now proper to use the molecular weight of 66,000 rather than 66,800 (see text).

to say, was to an extent prophetic in frankly asking the questions "What is *normal* hemoglobin?" and "How critical are existing criteria of protein purity and homogeneity?" (61). In their treatise of 1909 Reichert and Brown (35) called attention to many examples of alpha and beta crystalline forms of hemoglobin. Human hemoglobin, crystallized in the tetragonal system of symmetry by Drabkin's procedure, can be dissolved and then re-crystallized, according to Perutz *et al.* (70), in the orthorhombic configuration. In this case, the *environment* of crystallization appears to play a role. However, it is far from clear that all cases of dimorphism and polymorphism in crystallized hemoglobins are environmentally induced—a good subject for future study. Historically, I believe that Geiger (51) deserves the credit of being the first to have brought cogent evidence in support of the heterogeneity of normal adult hemoglobin. To what extent this heterogeneity may be co-linked with red cell heterogeneity (cf. 63) or with such environmental factors as aging of cells (71) remains to be clarified. This aspect of the problem will be covered by Drs. Kunkel and Morrison.

Concluding Remarks. Figure 5 was drawn in 1945 to depict the hemoglobin molecule and to illustrate the concept of partial molecular poisoning—a consequence of the four hemin structure (cf. 61). Despite the important recognition of the

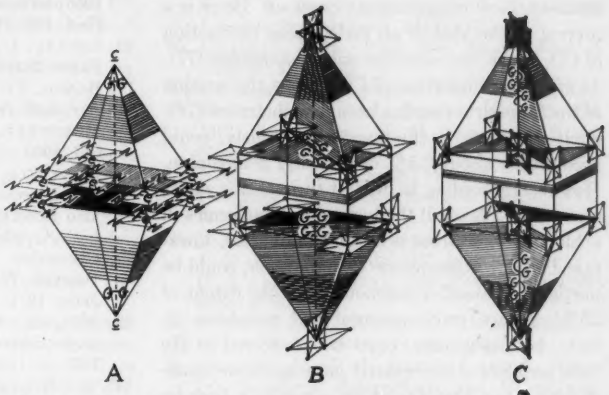


FIG. 6. Graphical projection of 'unit cell' of human hemoglobin in the frame of a bipyramidal tetragon. *A*, drawn to contain 2 molecules of hemoglobin per unit cell, is presumably outlawed. Alternatives *B* and *C* each contain 4 molecules per unit cell, hence 16 hemin residues. Structural diagrams conform to x-ray crystallographic findings of Perutz *et al.* (70) that there are 4 molecules per unit cell in tetragonal human hemoglobin, that there is internal symmetry in this system of crystallization, and that the asymmetric molecular weight unit is 34,000. In *C*, which is intuitively favored, the planes of the 16 hemins are parallel to each other, and 4 of the 16 hemins occupy a relatively internal position. *G*—*G* represents globin, each *G* being a 32,000 molecular weight unit.

many different hemoglobins, normal as well as abnormal, this diagram still remains consonant with known facts, including the latest information as to the orientation of the hemins, secured by the elegant technique of electron spin resonance (72). There is one change of non-spectacular nature. The molecular weight of hemoglobin, based on the iron content, is closer to an even 66,000 than 66,800 as shown. This comes about by virtue of the tentative acceptance of Drabkin's analytical value of 0.338% of iron in crystallized human hemoglobin (73) by the Protein Commission of the International Union of Pure and Applied Chemistry (July, 1955), thereby sanctioning the use of the one iron atom equivalent weight of 16,520 instead of the customary 16,700. It may now be reported that at long last the iron content of human hemoglobin has been determined directly on a dry weight basis, and it is in agreement with Drabkin's earlier analyses (74).

As biologists we must ask "To what extent does molecular abnormality in hemoglobin influence the major function of this substance?" Within the sensitivity of existing techniques of measuring oxygen dissociation, the answer thus far is "Not at all, in the case of sickle cell hemoglobin" (75). Perhaps Roughton need not worry, after all.

The molecular lesion, involving the protein portion of hemoglobin, was subtle, and difficult to ascertain. After 8 years—a short time in the history of hemoglobin—we seem to be on the threshold of understanding it. Probably the most significant clue in this connection is presaged in a report by V. M. Ingram (76). In figure 5, the horizontal line drawn through the oblate spheroid represents the splitting of hemoglobin into two equal halves in 4–6 molar urea. Perutz, I am sure, would be happy if some means were available to nicely divide the molecule into eighths or at least quarters, thereby simplifying it for study. Ingram had the fruitful thought of doing this by means of an enzymatic dissection with trypsin. This has disclosed that in sickle cell hemoglobin, in contrast with the normal, 1 polypeptide out of some 30 is positively charged. Furthermore, on subjecting the polypeptides of the two hemoglobins to chromatography, Ingram has found that "there is a difference in the amino-acid sequence in one small part of one of the polypeptide chains."

The lethal molecular disease, Sickle Cell Anemia, is truly amazing. One gene is responsible for a biochemical lesion in the hemoglobin molecule—a single amino acid sequence in one small part of the total complex is involved. As a result, the deoxygenated form of hemoglobin is insoluble and crystallizes within the red cell, distorting its structure, thereby condemning it to hemolytic destruction.

Figure 6 is in the nature of a postscript. It is an attempt to project in graphic form the 'unit cell' of human hemoglobin within the structural frame of a tetragon. No apology need be given for the extra-scientific character of this projection.

We still do not know fully what *normal* hemoglobin is like. It is obtained from a mixed population of cells, some of which are in their terminal

moment. New complications beset us. There is a revival of the idea of an endogenous production of CO, which may combine with hemoglobin (77). *In vitro*, the liberation of CO during the scission of the porphyrin ring has been demonstrated (78). Such a process, if effective endogenously, could account for about 0.5% of carbonyl hemoglobin. However, according to present theory, this would mean that 2% of all the hemoglobin molecules in a blood sample from a normal individual, unexposed to any atmospheric CO whatever, could be *partially poisoned* endogenously (to the extent of 25%). Hence, environmental and metabolic effects, besides genetic, must be considered in the total problem of molecularly heterogeneous hemoglobins. It is also timely to consider a broader base—the molecular heterogeneity of proteins in general. Indeed, Luetscher's finding that in nephrosis there is a shift in the ratio of two plasma albumins, one presumably being produced, under stress, in preference to the other (79), preceded by a number of years a similar deduction in regard to the rates of production of two hemoglobins, under the stress of anemia (63).

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INTERPRETATION OF ELECTROPHORETIC, SOLUBILITY AND DENATURATION DATA IN THE STUDY OF HEMOGLOBIN DIFFERENCES

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THE existence of a fetal and an adult form of hemoglobin in man and in a number of other species has been recognized for many years. The demonstration of an abnormal form of human adult hemoglobin in sickle cell disease, first reported at these meetings in 1949 (1), has led to a widespread search for abnormal hemoglobins in other anemias. By now at least nine abnormal forms of human hemoglobin[1], some of which are not known to be associated with disease, have been reported from widely scattered regions of the earth (2); and minor components have been isolated from the hemoglobin of normal individuals (3). It is not necessary for the purpose of the present paper to go into detail concerning the properties of the various forms, but certain identifying properties may be summarized. Fetal hemoglobin (hemoglobin F) differs from the others in its high resistance to alkali denaturation. Its ultraviolet spectrum and electrophoretic behavior are also different from those of the others. The major component of the hemoglobin of normal adults is called hemoglobin A. The minor components and abnormal hemoglobin all differ from hemoglobin A in electrophoretic mobility, and all of the abnormal hemoglobins differ from each other in mobility except hemoglobins S (sickle cell hemoglobin) and hemoglobin D, which are distinguished from each other by their solubility difference.

It is convenient to divide the sources of heterogeneity in the hemoglobin molecule to those associated with the heme or with the globin portions. Examination of the hemes prepared from the hemoglobins of different species and of hemoglobins A and S have not revealed any differences (4, 5), and it is likely that interspecies and intraspecies differences reside in the protein portions of the molecules, the globins. Thus, when we speak of genetically controlled differences in the properties of hemoglobin, we are referring to differences in the globins. It has been shown that hemoglobins A, S and C are produced under the control of genes that are transmitted as alleles (6), so that the abnormal forms are adult types. A homozygous individual has only one of these forms, and a heterozygous individual has two, and no more than two. Most of the other abnormal forms are probably controlled at the same locus, although complete genetic data are not yet available. The designation 'Hb' has been given to this locus (7). If more than two components are present in the same individual, these components may be derived from the two adult forms by chemical or physical processes, or other genetic loci may perhaps be involved. For example, an infant heterozygous for the adult forms has fetal hemoglobin in addition to two adult forms (8). Fetal hemoglobin may be present in addition to two adult forms in some forms of anemia (9). Partial conversion to ferrihemoglobin also results in electrophoretic inhomogeneity (10), but the difference in mobility between the ferrihemoglobins of two adult forms is the same as the difference between the corresponding oxyhemoglobins or carbonmonoxyhemoglobins. Presence of the same minor component in association with each of the adult forms, as is the case with fetal hemoglobin, suggests that the genetic locus responsible for this component is different from that for the adult globins. Figure 1 summarizes the genetic relationships of hemoglobins A, S and C in terms of electrophoretic analyses. Component X is probably the slow component (at alkaline pH) of Kunkel and Wallenius (3). This component is now designated A_2 (11), and its genetic control is probably independent of the Hb locus.

If the composition of the hemoglobin of the same individual changes with time, an alteration is occurring in his mechanism of hemoglobin synthesis. Such is the case in the infant, where the proportion of fetal hemoglobin decreases as he grows older (8), and in some acquired anemias, where fetal hemoglobin reappears in the adult

[1] The human hemoglobins are designated by letters by mutual agreement of interested investigators, as published in *Blood* 8: 386, 1953; 12: 90, 1957.

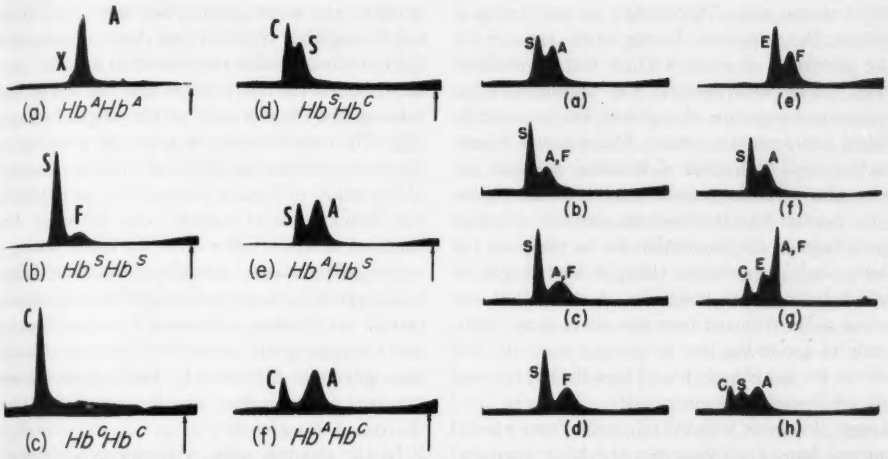

FIG. 1 (*left*). Electrophoretic patterns in cacodylate buffer of pH 6.5, Γ/2 0.1 corresponding to hemoglobin composition and genotypes in *a*) normal adult, *b*) sickle cell anemia, *c*) hemoglobin C disease, *d*) sickle cell-hemoglobin C disease, *e*, sickle cell trait, and *f*) hemoglobin C trait. Component X is probably the same as the A_2 component (11) reported by Kunkel and Wallenius (3). Fetal hemoglobin (F) may occur in anemias other than sickle cell anemia; its synthesis is apparently under the control of a genetic locus other than the Hb locus. From ITANO AND PAULING, *Svensk Kemisk Tidskrift*, in press.

FIG. 2 (*right*). Electrophoretic patterns of mixtures of some human hemoglobins in cacodylate buffer of pH 6.5, Γ/2 0.1. Hemoglobins A and F are of similar mobility, as are hemoglobins E and S; however, differences are detectable when each member of a pair is mixed with a third form. From (26).

(9). On the other hand, if different results are obtained from the same non-anemic individual from day to day, the accuracy of the analytical method is open to challenge since only 0.8% of the red cells of a normal individual is replaced each day.

The terms, homogeneity and heterogeneity, bear different implications to different investigators. The biochemical geneticist may consider the relationship of these terms to the one gene-one enzyme and template theories of the genetic control of protein biosynthesis. According to these theories, a given segment of a chromosome bears information regarding the amino acid sequence of the polypeptide constituents of a protein molecule, and this information is reflected in a template upon which the sequence of amino acids is determined (12). If such a mechanism is involved in the determination of the structure of a protein, how accurately is the information possessed by a gene transmitted in terms of the homogeneity of the protein which it controls? Are errors made in assembling the amino acids, so that the protein produced under the control of a single allele is heterogeneous? The physical chemist, on the other hand, is primarily interested in the end product of a fractionation procedure: is a protein prepared from individual or pooled samples according to certain specified methods a homogeneous entity consisting of identical molecules (13)?

It is evident that the question of hemoglobin heterogeneity is closely related to the genetic control of protein synthesis. This fact has been tacitly assumed in the past when protein samples have been identified according to species. Recent studies have shown that, in addition to hemoglobin, the β-lactoglobulin of cows (14) and the serum haptoglobins of man (15) exist in different inherited forms, not only in the same species, but in an individual as well. Thus, the mere statement that a protein of a given species is homogeneous or is heterogeneous is an ambiguous one. The population from which a protein sample is obtained may be heterogeneous with respect to the genetic locus corresponding to this protein; therefore, conclusions regarding heterogeneity, when based on pooled samples, provide no information as to the status of the protein synthesized under the control of a single allele. Even a sample from a single individual may have two components if he is heterozygous at the locus, and more than two components if other loci control other forms of the same protein. On the other hand, the crystalline material that is homogeneous may repre-

sent but one part of the protein as it occurs in a species, the remainder having been removed by the processes of concentration and crystallization. Finally, heterogeneity may appear after the amino acid sequence of a protein has been established under genetic control. Dimerization, dissociation, and hydrolysis of terminal peptides are examples of post-synthetic causes of heterogeneity. Normal human blood contains red cells that have been in the circulation for as many as 120 days, and degradative changes may begin to affect hemoglobin molecules in cells that are about to be removed from the circulation. Alteration of one of the four hemes per molecule, if it affects the net charge, would be sufficient to cause electrophoretic inhomogeneity. Lemberg and Legge (16) have isolated biliverdin from washed normal human erythrocytes and have suggested the possibility of the occurrence of intracellular alteration of hemoglobin.

The identification of a sample according to the species from which it was obtained is certainly inadequate in the case of hemoglobin. The hemoglobins of horse, sheep and cattle have been used extensively in physical and chemical investigations, and interspecies differences in solubility, electrophoretic mobility and resistance to denaturation have been known for many years. However, recent reports indicate that each of these species has two different forms of adult hemoglobin, some individuals having one or the other form, and others having both (17–19). It is entirely conceivable, then, for two laboratories to report divergent findings, in the same species, not only with regard to homogeneity but also with regard to physical properties and chemical composition, if they work with samples obtained from different animals.

All hemoglobin molecules are capable of undergoing certain specific reactions associated with their heme groups, and such reactions may induce physical heterogeneity in samples in which the globin portion is apparently homogeneous. Carbonmonoxyhemoglobin is a common contaminant in the blood of tobacco smokers and of individuals exposed to exhaust fumes, and ferrihemoglobin is produced in nitrite poisoning and in toxic reactions to certain drugs. The resistance to alkali denaturation of carbonmonoxyhemoglobin is greater than that of oxyhemoglobin (20), and the electrophoretic behavior of ferrihemoglobin is different from that of the ferrohemoglobin compounds (10). With respect to solubility behavior, carbonmonoxyhemoglobin, oxyhemoglobin, and ferrihemoglobin are similar, but they differ from ferrohemoglobin (21). Denaturation or conversion from one hemoglobin compound to another may take place in the course of an analysis and induce heterogeneity not present in the original sample (62). The determination of type and proportions of all components present is an important aspect of the study of human hemoglobin, and stroma-free hemolysates of washed cells are used. For chemical analyses that require the use of homogeneous preparations, samples from individuals homozygous for a given hemoglobin is used, and further purification is achieved by crystallization and electrophoretic separation. However, human hemoglobin is a relatively labile protein, and repeated crystallization may increase rather than decrease heterogeneity.

In the strictest sense a homogeneous protein preparation is composed of molecules in which both the amino acid sequences and spatial configuration of the peptides are identical. Electrophoretic studies showed that sickle cell hemoglobin carries about three more positive charges per molecule than normal adult hemoglobin, indicating a difference in content of ionizable groups (4). Solubility studies by Perutz and Mitchison confirmed the existence of a structural difference by demonstrating a vast difference in the solubilities of the two hemoglobins when deoxygenated (23). In spite of these striking differences in physical properties, the x-ray diffraction patterns of crystals prepared from the two forms were found by Perutz to be indistinguishable (24). Hemoglobin D is not separable from sickle cell hemoglobin by electrophoretic analysis, so the number and dissociation constants of their ionizable groups must be identical, or very nearly so. However, the solubility of ferrohemoglobin D is many times that of ferrohemoglobin S (25) and indicates the presence of a structural difference not detectable by electrophoretic analysis. These examples point out the limitations of physical and analytical methods in evaluating protein homogeneity or heterogeneity. On the other hand, electrophoretic analysis is capable of distinguishing two molecular species of hemoglobin that differ by a single charge (10), a difference that would be extremely difficult to demonstrate by amino acid analysis alone.

The purpose of this extended introduction has been to emphasize the importance of the genetic factors and of non-genetic alterations that contribute to the evaluation of differences in hemoglobin. Results that are not consistent with

genetic data or with the known reactions of hemoglobin must be evaluated with particular care. In some cases, an interpretation not dependent upon the assumption of heterogeneity is possible; in others, the data may furnish new information concerning either the mode of inheritance or intraerythrocytic alterations of hemoglobin.

INTERPRETATION OF DATA

Moving Boundary Electrophoresis. Zone electrophoresis has been widely used in recent years in the study of human hemoglobin. Dr. Henry Kunkel, who has been one of the pioneers in the development of this method, will cover this subject; therefore my remarks will be confined to the application of the moving boundary method to the study of hemoglobin. Hemoglobins A and S have the same molecular weight and shape (24), and their difference in electrophoretic mobility is independent of buffer at a given pH; their relative mobilities thus reflect differences in their content of ionizable groups and are not the result of dissociation, dimerization, or complexing with small ions. The other electrophoretically abnormal hemoglobins have not been characterized as thoroughly; however, the fact that they can be separated by independent methods suggests that they are distinct molecular entities. Two hemoglobins may differ in their content of charged amino acid residues and still have the same net charge at a particular pH, and a mixture of the two would appear electrophoretically homogeneous at this pH. Therefore, the evaluation of electrophoretic homogeneity necessitates analyses in several buffers of different pH (26).

In electrophoretic analyses of serum proteins, conditions are chosen such that enantiographic patterns are obtained. Since mobility differences in the electrophoresis are considerably smaller than those encountered in routine analyses of serum, such conditions result in poor resolution with some mixtures. In order to achieve good separation, conditions whereby sharpening of the ascending boundaries occurs at the expense of resolution in the descending boundaries are frequently used for the study of hemoglobin, and compositions are obtained by comparison with patterns obtained from known mixtures. In cacodylate buffer of pH 6.5 and ionic strength 0.1, the difference between apparent and true composition is not large (27). Hemoglobins A and S are well resolved with prolonged electrophoresis, as are S and C, which differ less in mobility than do A and S. On the other hand hemoglobins A and F are not resolved, nor are hemoglobins S and E. However, if A-S and F-S mixtures containing the same proportion of S are compared, it is evident that hemoglobin A migrates slightly more rapidly than F at this pH. Similar comparison of A-S and A-E mixtures shows that S migrates slightly more rapidly than E. These comparisons are shown in figure 2. The use of such mixtures circumvents the necessity of obtaining accurate values of absolute mobilities. The mean difference in net charge between A and F and between S and E at pH 6.5 is probably less than one. The demonstration of this difference by measurements of absolute mobility would be a difficult task.

More rapid separation can be obtained by the use of dilute buffers, and Hoch (28) demonstrated minor components in human adult oxyhemoglobin by electrophoresis in 0.01 M Na_2HPO_4. However, the discrepancy between apparent and true composition may become large under such conditions. The degree of resolution obtainable in dilute buffers is evident from our experiments in phosphate buffer of pH 6.85 and ionic strength 0.01. With use of this buffer intermediate compounds of hemoglobin that differ by a single charge were resolved in 5 hours or less, as shown in figure 3 (10).

Measurement of Solubility. It was shown many years ago that many crystalline proteins behave as homogeneous components with respect to solubility in salt solutions, and the phase rule was successfully applied to the interpretation of the solubility behavior of proteins and protein mixtures (29). In phase rule studies, increasing amounts of protein are added to a constant volume of salt solution kept at constant temperature, pressure, pH, and salt concentration (fig. 4). A homogeneous protein goes into solution until the solvent is saturated; thereafter, further addition of protein does not alter the concentration of protein in solution. A mixture of non-interacting proteins goes into solution in discrete steps and finally reaches constant concentration. An interacting mixture yields a curvilinear plot of concentration against amount of protein added. Reliable equi-

FIG. 3. Separation of intermediate compounds of carbonmonoxyhemoglobin and ferrihemoglobin by moving boundary electrophoresis in phosphate buffer of 6.85, Γ/2 0.01. From (10), by permission of *J. Am. Chem. Soc.*

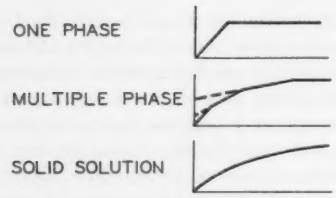

FIG. 4. Three types of solubility behavior of proteins in aqueous solvents of constant salt concentration. For details see (29).

librium solubilities are obtained with amorphous, as well as crystalline, hemoglobin as the solid phase. Mixtures of the human ferrohemoglobins have solubility relationships that indicate solid solution formation (30). Therefore, the solubilities of two different mixtures are not comparable unless the ratio of total hemoglobin to solvent is the same for both determinations.

Normal adult hemoglobin satisfies the phase rule criterion for a homogeneous protein when tested in phosphate buffer as crystalline carbonmonoxyhemoglobin or as amorphous ferrohemoglobin (21, 30). However, as earlier mentioned, more than one electrophoretic component has been detected in the hemoglobin of normal adults. Either the amount of heterogeneity is too small to be detectable by the constant-solvent method, or the solubility relationships of the components in the solvents used are such that heterogeneity is concealed. In some cases the detection of heterogeneity by this method requires the use of more than one solvent (31).

Another method for comparing solubilities is the variable-solvent or salting out method. The amount of protein and volume of solvent is kept constant, but the salt concentration is changed. The pH and temperature are also held constant. The logarithm of the solubility of a homogeneous protein is related to the ionic strength of the solvent by the equation $\log S = \beta - K_s(\Gamma/2)$, where $\Gamma/2$ is the ionic strength of the solution phase, β is a hypothetical solubility at zero ionic strength for a given pH and temperature, and K_s is characteristic for a protein and solvent but independent of pH and temperature (32). The salting-out curves of carbonmonoxyhemoglobin A in phosphate buffer has several discontinuities and it has been suggested that each discontinuity results from a different hemoglobin component (33). However, such behavior can be due to phase transitions in the solid phase and not to heterogeneity (30, 34). There is support for this hypothesis in the work of Perutz (24), who observed that the cyrstal form of hemoglobin A differs at different phosphate concentrations. Thus, it may be that the validity of the salting-out equation as a test for homogeneity is restricted to each solid phase of a homogeneous protein, and that each phase is characterized by a different β and K_s. Ogston and Tombs (34) have proposed a method for testing whether a discontinuity is due to a phase transition or to heterogeneity. The test, as indicated by figure 5, consists of increasing the amount of protein added to each of the equilibrium mixtures and observing whether the salt concentration at which the discontinuity occurs is shifted. The occurrence of a shift would indicate heterogeneity unless interaction between components occurs.

Measurement of Rate of Denaturation in Alkaline Solutions. Differences in the rate at which hemoglobins are denatured at high pH have been used to evaluate their homogeneity or heterogeneity. Oxyhemoglobin is denatured in alkaline solution to form denatured alkaline globin ferrihemochromogen. The reaction may be followed either by observing the rate of change in solubility in neutral salt solution or at the rate of change of absorbance of an alkaline solution (35). The results are used, not only to detect heterogeneity, but also to estimate the proportions of different components present. The assumptions involved in the interpretations of the results are *1*) the denaturation reaction is first-order with respect to concentration for each hemoglobin component; *2*) the absorption spectrum of the native and denatured protein is the same for each component; *3*) loss of solubility and change of spectrum are simultaneous processes; *4*) the soluble phase and the precipitate contain only native and denatured hemoglobin, respectively; and *5*) pH remains constant during the reaction. In most cases the denaturation of a homogeneous sample is consistent with these assumptions and follows a simple exponential course at constant pH.

$$(A_t - A_D)/(A_N - A_D) = e^{-kt}$$

A_t, A_N, and A_D are the absorbances (or solubilities) of the reaction mixture at time t, of oxyhemoglobin and of denatured alkaline globin ferrihemochromogen, respectively; and k is the first-order rate constant (including the constant hydroxide ion concentration). If the assumptions

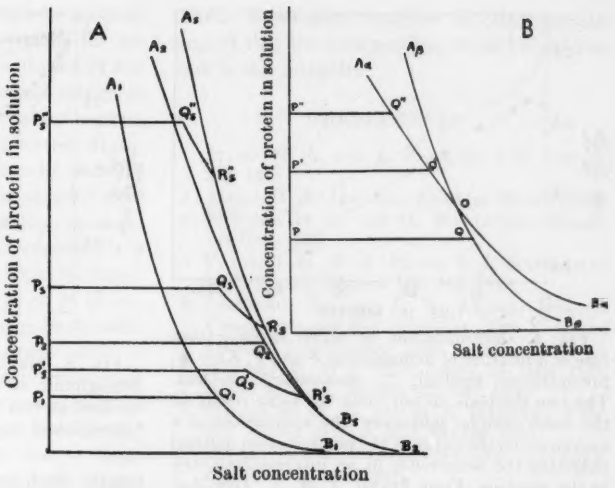

FIG. 5. Variable-solvent solubility test for protein heterogeneity. *A*, discontinuities resulting from heterogeneity. *B*, discontinuity resulting from change in the nature of the solid phase of a homogeneous protein. From (34) by permission of the authors and the editors of *Nature*.

as previously stated are valid, a mixture containing two components denaturing at different rates follows a course represented by the sum of two exponentials, and the same plot is obtained at any wavelength.

$$(A_t - A_D)/(A_N - A_D) = (1 - X)e^{-k_1 t} + Xe^{-k_2 t}$$

Here k_1 and k_2 are the rate constants for alkali denaturation of the two components, and $1 - X$ and X are their respective mole fractions. When k_1 is large compared to k_2, the first term becomes negligible after a short time, and thereafter the semilogarithmic plot of the reaction becomes a straight line. The slope of this line is k_2, and the extrapolated value of its ordinate at zero time is log X. In the study of human hemoglobin the alkali denaturation reaction is useful for the identification and quantitation of fetal hemoglobin, which denatures much more slowly than the normal and abnormal adult forms.

The mechanism can be tested by stopping the reaction before completion and separating the soluble and insoluble fraction. If the reaction mixture contains two hemoglobin components, the soluble fraction should have an increased proportion of the more resistant component. Mixtures of fetal hemoglobin and adult hemoglobin show this behavior, and adult hemoglobin can be removed from a mixture by alkali denaturation (9).

The percentages of fetal hemoglobin obtained by the photometric and precipitation methods do not agree (36). The probable reason for the discrepancy is that the properties upon which the respective methods are based do not change together; insoluble molecules may contain one or more hemes with the native oxyhemoglobin spectrum, and soluble molecules may contain hemes with the ferrihemochromogen spectrum. Coprecipitation of undenatured molecules with the insoluble precipitate may introduce another error. Different results obtained with use of the two methods are shown in figure 6.

An ambiguity arises in the interpretation of alkali denaturation data since the integrated rate equation for two successive unidirectional first-order reactions has the same form as that for the sum of two independent first-order reactions.

$$(A_t - A_D)/(A_N - A_D) = (1 - Y)e^{-k_1 t} + Ye^{-k_2 t}$$

$$Y = [k_1/(k_1 - k_2)][(\epsilon_I - \epsilon_D)/(\epsilon_N - \epsilon_D)]$$

In the above equations, k_1 and k_2 are the rate constants of the reaction from native protein to intermediate and from intermediate to denatured protein, respectively. ϵ_N, ϵ_I, and ϵ_D are the molecular extinction coefficients of the native, intermediate, and denatured forms, respectively.

Since the semilogarithmic plot of the alkali denaturation of hemoglobin A does not follow a linear plot, it has been inferred that the protein contains a resistant and a labile component (37). The proportions obtained by analyses of such plots do not, however, agree with electrophoretic analyses. Betke (38) found that, unlike mixtures of hemoglobins A and F, samples of hemoglobin

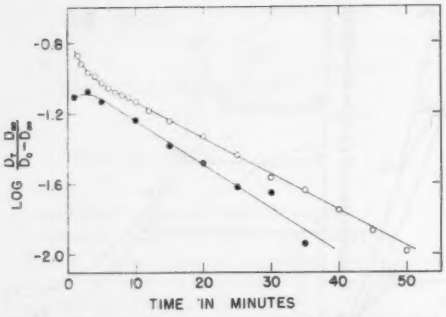

FIG. 6. Determination of alkali denaturation rate of a mixture of hemoglobins S and F. *Key:* ●, precipitation method; ○, photometric method. The two methods do not yield the same result on the same sample; moreover, the appearance of a maximum on the curve of the precipitation method indicates the occurrence of an intermediate step in the reaction. From ITANO, *A. M. A. Arch. Int. Med.* 96: 287, 1955.

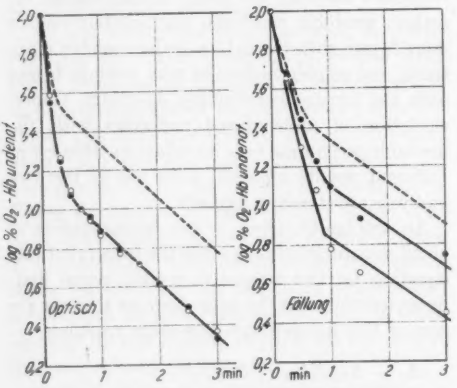

FIG. 7. Test of alkali denaturation reaction as a method for fractionating normal adult hemoglobin. *Left*, photometric method; *right*, precipitation method. The denaturation reactions were stopped before completion. The soluble residue was reexamined by the same method. *Dotted lines* indicate the expected course if the non-linearity of the original curves were due to heterogeneity. *Key:* ○, original curve; ●, curve of soluble residue. From (38).

A were not separable into a labile and a resistant fraction by partial denaturation. The results obtained by Betke are shown in figure 7. If an intermediate product appears in the course of the reaction, it is unlikely that the ratio $(\epsilon_I - \epsilon_D)/(\epsilon_N - \epsilon_D)$ would be the same at all wavelengths. The postulated mechanism can thus be tested by observing the reaction at more than one wave-

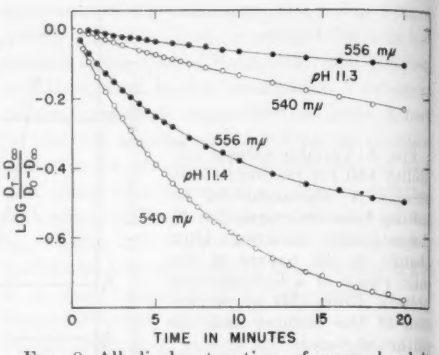

FIG. 8. Alkali denaturation of normal adult hemoglobin as determined by the photometric method at two wavelengths and two pH values. Unpublished experiments of the author.

length. Such experiments by the author have shown that more than one step is involved in the alkali denaturation of normal adult hemoglobin (fig. 8).

Comparison by Two or More Independent Methods. The validity of an observation of heterogeneity by one method can be checked in some cases by the application of another. For example, Huisman and Prins (39) have shown that mixtures of human hemoglobin separate in column chromatography is the same proportions as in electrophoresis. Others have shown that the proportion of fetal hemoglobin in a mixture is, within the accuracy of the methods, the same whether determined by alkali denaturation, electrophoresis, or ultraviolet spectrophotometry (40, 41). In comparing the results obtained by two independent methods, one must be aware of the effect of the presence of four hemes in each molecule of hemoglobin. A sample that contains 98% carbonmonoxyhemoglobin and 2% ferrihemoglobin spectrophotometric analysis may reveal two components in the ratio of 92% to 8% by electrophoretic analysis. The reason for this apparent discrepancy is that the oxidized hemes are distributed one to a molecule, each affected molecule having three carbonmonoxyhemes and one ferriheme.

CONCLUSIONS

All of the abnormal hemoglobins reported to date are electrophoretically abnormal. This situation does not, however, warrant the conclusion that all mutations affect the net charge of a protein. More samples have been examined by electrophoresis than by any other method. In spite

of the sensitivity of the electrophoretic method, the substitution of one charged residue for another with the same pK, the substitution of one uncharged residue for another, and alterations in the relative positions of the same charged residues without change in pK are not detected. Mutations that do not affect electrophoretic mobility are undoubtedly being missed. If samples were screened as thoroughly by solubility measurements as they have been by electrophoresis, it is likely that other inherited forms would be found.

The ultimate question that remains to be answered is whether or not the hemoglobin synthesized under the control of a single allele is homogeneous. An affirmative answer can be given with respect to electrophoretic homogeneity of the major components of human hemoglobin; homozygous individuals have only one of these, and heterozygous individuals have two. Hemoglobin A, the major component of the hemoglobin of normal adults, is the only protein that has satisfied the steady-state criterion for electrophoretic homogeneity (28). Hemoglobins A, S, and C are homogeneous in phosphate buffer of pH 6.85 and ionic strength 0.01, in which the effect of a single charge difference on electrophoretic mobility is detectable (42). Minor components are discernible in both buffers; however, they are not distributed symmetrically about the major component, as would be the case if heterogeneity resulted from random errors in the incorporation of charged amino acids. Evidence for greater heterogeneity based on alkali denaturation and variable solvent solubility data is not conclusive since these data are subject to alternative interpretations. If minor components are produced under genetic control, the locus or loci involved are probably different from that which controls the synthesis of hemoglobin A and its mutant forms, S, C, etc. Heterogeneity may also result from post-synthetic alterations of the molecule, either intracellular degradation or alterations that occur in the course of preparation and analysis of the sample. The latter possibility should receive serious consideration when the degree of heterogeneity shown by one method cannot be confirmed by an independent method. With respect to the incorporation of charged residues, the available evidence thus indicates that the translation of genetic information is accurate. In the absence of sequential analyses of hemoglobin, a similar statement with regard to the uncharged residues in a hemoglobin controlled by a single allele must be withheld; however, the results of complete analyses of other proteins suggest that the incorporation of uncharged residues is also accurate.

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DISCUSSION: CONFORMATIONAL DIFFERENCES OF MERCAPTO GROUPS OF FOUR HEMOGLOBINS[1]

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AN INTERPRETATION of the data obtained by the argentometric-amperometric and mercurimetric-amperometric titrations on hemoglobin solutions in the study of conformational differences with respect to the titratable mercapto groups of hemoglobins will be presented. Evidence for the molecular heterogeneity of four hemoglobins with respect to the conformation[2] of sulfur atoms (of —SH groups) will be introduced. The data obtained by the argentometric-amperometric titration method indicate that the maximal number of titratable sulfhydryl groups is the same for Hb-A, Hb-S, and Hb-C, whereas, the mercurimetric-amperometric titration data vary. This suggests that the spatial arrangements (conformations) of sulfhydryl groups are different in these hemoglobins. On the other hand, the normal fetal hemoglobin titration data indicate that there is a quantitative as well as conformational difference from the normal adult hemoglobin molecule with respect to the titratable mercapto groups.

Pauling (1, 2) suggested that the difference in structure between a 'normal' and an 'abnormal' hemoglobin molecule may be a difference in the way in which the polypeptide chains are folded. The amino acid composition of Hb-A, Hb-S and Hb-C are reported to be similar (3–5). Ingram (6) has shown that there is one peptide which is uniquely different in the tryptic digest of sickle cell hemoglobin—the 'sickle cell' peptide. Furthermore, he has shown that there is one amino acid which is different from the corresponding one in the normal hemoglobin molecule. This finding does explain the electrical charge difference between the normal and sickle cell hemoglobins. In addition to Ingram's finding, there may be peptide conformational differences among the hemoglobins, as suggested by the mercapto groups' spatial arrangements (which is the main theme of this paper).

When the folding or coiling of the polypeptide chains of a protein molecule is changed, the

[1] This work was aided in part by funds supplied by the Office of Naval Research and the Bureau of Medicine and Surgery, Dept. of the Navy, and done under contract between the Office of Naval Research and the Univ. of Pennsylvania. This work was initiated by the author at the California Institute of Technology in 1954; the study of Hb-A and Hb-S was completed at Pasadena in 1956, which resulted in Contribution No. 2175 from the Institute.

[2] The word 'conformation' was first used by W. N. Haworth (1929) in *The Constitution of Sugars*, which can be defined in several ways. One of the most general definitions is as follows: the conformations of a molecule are those arrangements in space of the atoms of the molecule which are not superposable upon each other. The word constellation', of more recent origin (1932), by Ebel (in *Stereochemie*, edited by Freudenberg), is used in the same sense and in the literature of chemical physics the term 'rotational isomer.'

chemical constitution is not altered, since it is determined by the number and sequence of amino acids. A new term had to be coined for changes of this type: Kuhn (7) designated the definite arrangement of the peptide chains as a 'constellation'; furthermore, Lumry and Eyring (8) used the equivalent term 'conformation'.[2] As stated above, titration data will be presented which suggest that there are conformational differences among human hemoglobins; the differences in the spatial arrangement of —SH groups suggest corresponding differences in the peptide conformations.

MATERIAL AND METHODS

The hemoglobins were crystallized by the method of Drabkin (9, 10); the crystals were dissolved in water and then dialyzed against a large volume of de-ionized water for about 12–18 hours before use. The fetal hemoglobin was 'purified' from the cord blood by two methods: *a*) an alkali denaturation method of Chernoff (11), *b*) a chromatographic method of Boardman and Partridge (12) as modified by Huisman and Prins (13). The fetal hemoglobin in crystalline form was obtained in both instances after the excess water was removed by evaporation from the viscose sausage casing which was placed in front of an electric fan. When the volume of the hemoglobin solution was reduced nearly to the original volume, it was placed in 2.8 M phosphate buffer in the usual manner. This gives rise to refractile granules; they are separated by centrifugation. The refractile granules are dissolved in a minimal volume of 2.8 M phosphate buffer at 0°, and the solution is placed in a cold room at about 7° where it is allowed to crystallize. The crystals thus obtained appear to be of pseudo-hexagonal type, and they are essentially the same in appearance as those shown in a photomicrograph of Jope and O'Brien (14).

The hemoglobin concentration was determined spectrophotometrically. After an aliquot was saturated with carbon monoxide, its optical density was determined in a 1-cm Corex Beckman cell at 540 mμ in the Beckman Model DU or B spectrophotometer. A molecular extinction coefficient of 14.7×10^3 at 540 mμ and a molecular weight of 16,700 per heme were adopted for the calculations (15). The hemoglobin concentration in grams per liter is 1.14 × optical density × dilution factor.

TABLE 1. BINDING OF SILVER AND MERCURY BY 4 DIFFERENT HUMAN HEMOGLOBINS

	Amperometric Titration Data		No. of —S—Hg—S— Linkages
	Ag/4Fe	Hg/4Fe	
Hb-A	8.01 ± 0.27	5.89 ± 0.16	2
Hb-S	7.91 ± 0.41	5.02 ± 0.36	3
Hb-C	8.06 ± 0.20	8.02 ± 0.31	0
Hb-F	6.04 ± 0.25	3.20 ± 0.60	3

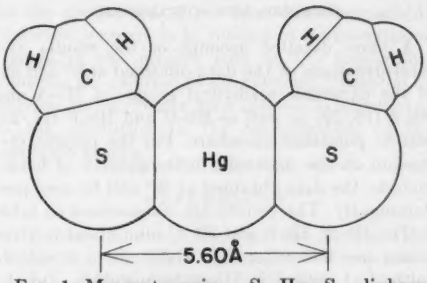

FIG. 1. Mercapto pair, —S—Hg—S— linkage. Centers of sulfur atoms are separated by twice the convalent Hg–S bond distance, giving —S—Hg—S = 5.60A. The beta carbon atoms (shown as CH_2's) are bonded to the sulfur atoms, which in turn are bonded to the alpha carbon atoms in the polypeptide chain.

The apparatus[3] and technique used for the amperometric-titrations are essentially the same as previously described (16, 17). The argentometric-amperometric method was that of Benesch, Lardy, and Benesch (18); the titrations were performed in 30 ml of 'Tris' buffer, pH 7.4. The solution was thermostated in a cell similar to the one described by Neilands and Cannon (19) modified by the use of a 50-ml Pyrex beaker as a 'lining.'

The mercurimetric-amperometric titration method was that of Kolthoff, Stricks and Morren (20); phosphate buffer, pH 7.3 in the presence of 0.5 M KCl was used. Pure nitrogen was bubbled through the solution to remove oxygen. After the buffer was deoxygenated, hemoglobin solution was then added from a self-adjusting micropipette (21). The titrating agents were added from a syringe microburette[4] as previously described (16, 17). The results were calculated graphically by plotting the current against the volume of the titrating agent in the usual manner; from the equivalent point in the titration, the number of heavy metals bound per molecule of hemoglobin was calculated.

[3] An automatic amperometric titration apparatus of the author is available; however, it was not used for this investigation. It is described in *Encyclopedia of Instrumentation for Industrial Hygiene*, edited by C. D. Yaffe, K. H. Byers, and A. D. Hosey. Ann Arbor, Mich: Univ. of Michigan Press, 1956, p. 430.

[4] 'AGLA' syringe microburette of Burroughs Wellcome and Co., Tuckahoe, N. Y.

RESULTS AND CONCLUSIONS

A more detailed account of the results and interpretations of the data obtained at 0° and 38° of the titratable sulfhydryl groups of Hb-A and Hb-S (16, 22) as well as Hb-C and Hb-F (17, 23) will be published elsewhere. For the present discussion on the molecular heterogeneity of hemoglobins, the data obtained at 38° will be used predominantly. The results are summarized in table 1. The Hb-A, Hb-S and Hb-C bind about 8 silver atoms per molecule; thus there are 8 titratable sulfhydryl groups in these hemoglobins. On the other hand, the normal fetal hemoglobin binds about 6 silver atoms per molecule; thus there are 6 titratable —SH groups in Hb-F.

The mercurimetric-amperometric titration data vary (see table 1). At 38°, dialyzed normal adult hemoglobin binds about 6 mercury atoms per molecule. It is highly probable that two —S—Hg—S— linkages are formed. This mercury-capturing linkage is diagrammatically shown in figure 1. In —S—Hg—S— the centers of the sulfur atoms are separated by twice the covalent Hg—S bond distance, giving S—Hg—S = 5.60 Å. To each of the sulfur atoms is bonded the beta carbon atom (shown as CH_2 in fig. 1), which in turn is bonded to the alpha carbon atom in the polypeptide chain. Thus, it can be seen that the cysteine residues are essentially afixed to the polypeptide chain. This fact makes the interpretation of the mercapto conformational differences more significant. On the other hand, the concept of 'mobile' mercapto groups would make the interpretation of conformational differences of —SH groups in a molecule meaningless. In Hb-A there are four —SH groups that are close enough (two and two) to be handled by just 2 mercury atoms and that the other two pairs of —SH groups are separated too far. This is schematically represented in figure 2, Hb-A. In the schematic diagram, there are 4 'lone' —SH's represented at the periphery of the drawing; these are titratable at 0°—at 0° Hb-A binds 4 Ag as well as 4 Hg atoms per molecule (16).

Dialyzed sickle-cell hemoglobin binds about 5 mercury atoms per molecule at 38° (see table 1). In accordance with the previous picture, this would mean that 3 —S—Hg—S— linkages are possible in this molecule. This suggests that 6 —SH groups are so paired as to be able to bind 3 mercury atoms and that the other pair is separated too far. Thus, there are 2 'lone' —SH groups in the sickle cell hemoglobin molecule and 3 pairs of mercapto groups. This is schematically represented in figure 2, Hb-S. At 0° Hb-S binds 4 Ag atoms but on the other hand it binds 3 Hg atoms per molecule (16); thus —SH groups are represented at the periphery of the diagram as 1 mercapto pair and 2 'lone' —SH's.

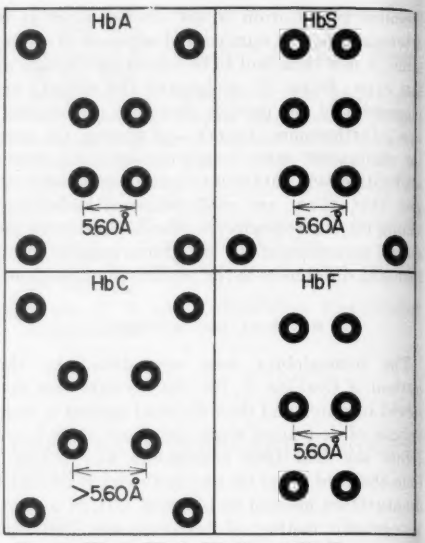

FIG. 2. Hb-A, Hb-S, Hb-C and Hb-F.
Hb-A, a conformation of sulfur atoms (of titratable —SH groups at 38°) of normal adult hemoglobin molecule; *Hb-S*, a conformation of sulfur atoms (of titratable —SH groups at 38°) of the sickle cell hemoglobin molecule; *Hb-C*, a conformation of sulfur atoms (of titratable —SH groups at 38°) of the C hemoglobin molecule; *Hb-F*, a conformation of sulfur atoms (of titratable —SH groups at 0° and 38°) of the normal fetal hemoglobin molecule.

Dialyzed hemoglobin 'C' binds 8 mercury atoms as well as 8 silver atoms per molecule. The data suggest that Hb-C contains no mercapto pair and that the 8 —SH groups are 'lone' sulfhydryl groups as schematically represented in figure 2, Hb-C. At 0° Hb-C binds 4 silver and 4 mercury atoms per molecule, as does Hb-A; consequently the sulfur atom conformation is the same for Hb-A and Hb-C at 0° (17).

The normal fetal hemoglobin binds 6 silver atoms and 3 mercury atoms per molecule at 38° as well as at 0°. Thus, the data suggest that 3 —S—Hg—S— linkages are formed. There are 3 mercapto pairs in the fetal hemoglobin molecule. This is shown schematically in figure 2, Hb-F.

It can be seen from the schematic diagrams in figure 2, Hb-A, Hb-S, and Hb-C that the maximal number of titratable sulfhydryl groups is the same in these molecules, however, there are conformational differences. There are 2, 3, and 0 mercapto pairs in Hb-A, Hb-S, and Hb-C, respectively. In Hb-F there are 6 titratable —SH groups and they are spatially arranged as 3 mercapto pairs; there are quantitative as well as conformational differences between Hb-A and Hb-F.

DISCUSSION

Some of the —SH groups appear to be arranged in pairs so that they are able to bind one mercury atom per pair of mercapto groups forming an —S—Hg—S— bridge. Ingram (24, 25) suggested that the results he obtained with mercurimetric-amperometric titration of native and denatured hemoglobin showed that one $HgCl_2$ molecule can effectively block two argentometrically titratable —SH groups. Hughes (26) was led to a similar conclusion by the finding that mercaptalbumin which has one —SH group per molecule can form a dimer, $Hg—(S—pr)_2$, with $HgCl_2$.

Additional evidence for the pairing of —SH groups comes from x-ray diffraction data. Studies of Perutz (27) and Perutz, Liguori and Eirich (28) have shown that the molecules of horse and human hemoglobin possess a diad axis of symmetry. Ingram (24) believed that his finding of an even number of titratable —SH groups agrees well with this symmetry, and accordingly concluded that native ox and human hemoglobins appear to have 4 —SH groups in two pairs. Furthermore, x-ray diffraction measurements of horse hemoglobin with 4 equivalents of silver showed only one slightly elongated peak in the electron density map for each pair of silver atoms, indicating directly that the two —SH groups must be close together.

More recently Riggs and Wolbach (29), using several mercurials on horse hemoglobin, examined further the nature of heme-heme interactions and their dependence upon —SH groups of the protein and similarly concluded that —S—Hg—S— linkages are formed; this linkage reduces the heme-heme interaction in horse hemoglobin. When —S—S— linkages are formed, similar reduction in heme-heme interaction takes place (30). Thus, hemes and —SH groups are closely associated; mercurials affect the hemoglobin spectrum to a small extent, and they affect n of Hill's equation and also affect oxygen affinity (29, 30).

It is now generally accepted that there is a close relationship between the genetic structure of an individual and the kind of protein he produces, and the kind of hemoglobin is dependent upon the spatial arrangement (conformation) of the peptide chains. Thus, the hemoglobin differences may be related to the way in which the peptide chains are folded (1, 2). This change in the folding of the peptide chains may be related to the conformational differences of the —SH groups. The mutated gene, in the instance of hereditary anemia leading to the production of an 'abnormal' hemoglobin molecule, appears to be related to conformational change in the protein part of the hemoglobin molecule (in addition to the evidence presented by Ingram, ref. 6); this in turn appears to be reflected in the alteration in the conformation of the mercapto groups.

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MINOR HEMOGLOBIN COMPONENTS OF NORMAL HUMAN BLOOD

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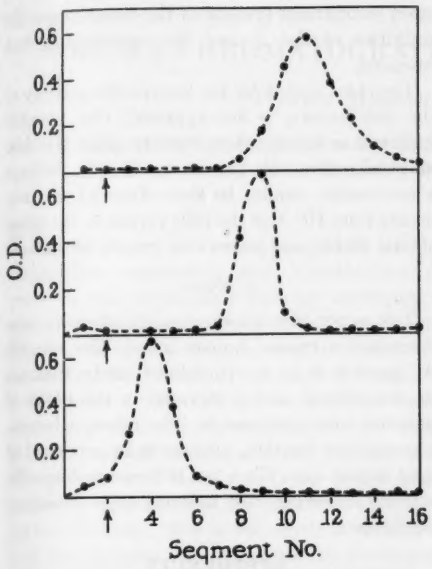
IN 1946 Stern and Reiner (1) noted several minor components in electrophoretic analyses of human red blood cell hemolysates by the use of the classical Tiselius procedure. This method did not permit conclusions as to whether these represented hemoglobins or non-colored protein constituents of the red blood cell. Derrien and his associates (2), Hoch (3), Shavit and Brewer (4) and, more recently, Berry and Chanutin (5) have presented evidence for the heterogeneity of human hemoglobin through the use of low ionic strength buffers for electrophoresis, particularly sodium cacodylate. Secondary components at relatively high concentration were observed. Isolation has not been achieved thus far and the exact significance of the large subfractions remains to be determined. It seems possible that they may be related to the fractions recently separated chromatographically by Morrison and Cook (6).

In a previous report (7) evidence was presented for the presence of two minor hemoglobin components in normal human blood. The one, present at a concentration of approximately 2.5% was a clearly defined entity differing markedly in charge from the major Hb A and resembled the abnormal hemoglobin, Hb E, in mobility. The other component was a heterogeneous fraction, migrating faster than Hb A, but with which it overlapped considerably. It could not be quantitated. Further work has substantiated the observation that these two fractions are distinctly different from Hb A.

A photograph of the appearance of the red color of the hemoglobins in the hemolysate from the blood of a normal individual is shown at the bottom of figure 1. A small but distinct component (A_2) migrating more slowly than Hb A is visible in this experiment, carried out by zone electrophoresis on a thin starch slab in barbital buffer pH 8.6, ionic strength 0.1. In addition, a small amount of material migrating faster than Hb A is just visible as a pointed projection in front of the main A spot. Isolation of these minor fractions was possible through the use of broad starch blocks which accommodated as much as 20 cc of an 8 % hemoglobin solution. Following concentration by ultrafiltration, these fractions showed their original mobility in a second electrophoretic separation. The upper curves in figure 1 show the patterns of the three fractions isolated from normal hemoglobin. The lower curve represents the slowly migrating A_2 component; the middle, Hb A and the top, the broad heterogeneous fast fraction. The three fractions were studied in this experiment under identical conditions on the same starch block. The original hemoglobin sample prior to isolation of the subfractions is also shown in figure 1. The mobilities of the isolated fractions are similar to those of the corresponding fractions in the whole hemoglobin solution.

Similar components with the same relative mobilities were obtained regardless of the hemoglobin derivative employed. Oxyhemoglobin, carbonmonoxyhemoglobin, methemoglobin, cyanmethemoglobin and reduced hemoglobin all showed a similar distribution despite differences in over-all mobility. Different anticoagulants and different procedures of laking the red blood cells gave similar results. Numerous observations with the isolated A component indicated that it did not give rise to the A_2 fraction. However, considerable evidence was obtained that the fast fraction or material like it could be produced from Hb A. This transformation occurred slowly with oxy- and carbonmonoxyhemoglobin but was particularly rapid when methemoglobin or cyanmethemoglobin was used. Old hemoglobin samples also were found to contain considerably more of the fast fraction. Though the fast fraction could not be quantitated it appeared to be present at a concentration somewhere between 4 and 12 % in fresh hemoglobin specimens. The accumulated evidence strongly suggested that the A_2 component was a well-defined entity, but that the fast fraction could well be derived from Hb A.

Quantitation of the A_2 component could be carried out. Replicate analyses of one normal hemoglobin sample separated 16 times showed a mean value of 2.51% with a standard deviation of ± 0.31. Examination of the blood of 65 normal



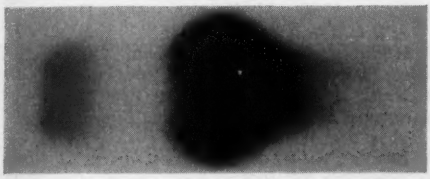


FIG. 1. Curves obtained following electrophoresis of isolated fractions from normal human hemoglobin. The fast fraction is at the top, the main A Hb in the middle, and A_2 at the bottom. The pattern of the original sample is also shown. Protein analyses by the modified Folin procedure. Arrow indicates site of application, anode is at the right.

individuals showed that the A_2 level was 2.54% ±0.35. Several hundred normal individuals have now been studied and the A_2 level appears to be very constant. The upper limit of normal is approximately 3.3%. In patients with thalassemia considerable elevation was frequently found. The highest level observed was 11% in an adult with an intermediate type of thalassemia. Four such individuals were studied and all showed levels between 8 and 11%. Ordinary thalassemia minor cases usually showed approximately twice the normal values. The over-all results in patients with thalassemia, excluding the Cooley's type, (34 cases) was 5.11% ±1.36. The infants with Cooley's anemia frequently had relatively normal levels. Two patients with thalassemia minor also showed levels in the normal range. The significance of the elevation in thalassemia was not apparent. No elevated values were obtained in any other condition studied. A compensatory increase of A_2 Hb similar to that of fetal hemoglobin in the presence of defective Hb A synthesis in this disease is a possible explanation. Elevation of the A_2 level in other conditions has not been observed. The details of these studies have been published separately (8).

In order to study further the significance of the A_2 and fast hemoglobin fractions, their specific activity was studied after administration of Fe^{59}. Observations by Schapira and associates (9) had indicated that this method showed heterogeneity for human hemoglobin. It was also thought that such experiments would provide evidence regarding the presence of the minor fractions in all red blood cells or just in selected cells at high concentration. Table 1 shows representative results obtained in two individuals at different times

TABLE 1. COMPARISON BETWEEN SPECIFIC ACTIVITY OF VARIOUS HEMOGLOBIN FRACTIONS AT DIFFERENT TIMES AFTER ADMINISTRATION OF Fe^{59}

Subj.	Days After Fe^{59}	Hb Fract.	% of Total	Spec. Act., cts/100 γ Fe	Ratio of Spec. Act.
M. F.	4½	fast	5.2	152.	.584
		A	92.3	296.	1.0
		A_2	2.5	282.	.95
	11	fast	4.8	208.	.49
		A	93.1	425.	1.0
		A_2	2.1	390.	.92
	21	fast	4.2	198.	.56
		A	93.2	354.	1.0
		A_2	2.6	297.	.84
	7	$fast_1$	3.1	145.	.32
		$fast_2$	13.6	397.	.87
		A_f.	51.4	420.	.92
		A_{s1}.	30.4	455.	1.0
		A_2	1.6	446.	.9
Y. R.	14	$fast_1$	1.2	178.	.43
		$fast_2$	3.7	232.	.56
		A_f.	59.4	353.	.85
		A_{s1}.	33.6	417.	1.0
		A_2	2.3	333.	.80
	37	$fast_1$	2.2	127.	.64
		$fast_2$	4.5	179.	.89
		A_f.	51.1	220.	1.1
		A_{s1}.	40.8	200.	1.0
		A_2	1.4	180.	.9

after the intravenous injection of approximately 32 μc of Fe^{59} (0.024 mg Fe). Two hospital subjects who did not appear to suffer from any hematological disorder were used in this study. In the experiments with patient *M.F.* the separated hemoglobin was divided into three fractions, designated A_2, A and fast. With the hemoglobin of patient *Y.R.* further subdivision was carried out. The fast material was divided into two parts, a very fast fraction (designated $fast_1$) and a relatively slower fast fraction, (designated $fast_2$). Hb A was also divided into a fast and slow portion. In each case, all of the separated hemoglobin fell into one of these fractions and the relative percentage of each in the different experiments is indicated in table 1. The radioactivity was determined in a well-type scintillation counter after the hemoglobin solutions had been brought to approximately equal concentrations as indicated by cyanmethemoglobin color. Iron analyses were carried out in duplicate on each solution after measurement of radioactivity. The most striking finding was the low specific activity of the fast fraction compared to that of Hb A. This was evident in all experiments, and can be seen best from the relative specific activity calculated by assigning the value 1.0 to either the whole of Hb A or to its least contaminated slower portion. Similar differences were observed when the radioactivity was measured in terms of the heme derivative. The experiments with the second patient are the more informative because the $fast_1$ fraction was completely free of Hb A. The $fast_2$ fraction, as well as the whole fast fraction in the experiments with the first patient, probably contained some Hb A, and thus the specific activity represented an average of the overlapping fractions. Hb A_2 showed a specific activity similar to but always slightly below Hb A. However, it was somewhat difficult to make an exact comparison because of the range in specific activities of the A and the overlapping fast fractions.

The exact reason for the low specific activity of the fast fraction is not apparent. One possible explanation would appear to be that the fast material is primarily present in old cells. Perhaps a conversion, similar to that observed *in vitro*, occurs from Hb A as the cells persist in the blood stream and are subjected to a variety of stresses.

SUMMARY

Two minor hemoglobin components have been described in normal human blood. The one, Hb A_2, appears to be a well-defined entity that can be quantitated and is elevated in the blood of patients with thalassemia. The other, a heterogeneous fast fraction, appears to be produced at least in part from Hb A but differences in specific activity following Fe^{59} indicate some biological significance.

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COLUMN CHROMATOGRAPHY OF HUMAN HEMOGLOBINS[1]

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OF THE many procedures employed for the purification, separation and identification of proteins, one of the most recently developed is ion exchange chromatography. Early work employing this method demonstrated its remarkable sensitivity. For example, the earliest work (1) with hemoproteins employing ion exchange resins demonstrated that reduced cytochrome *c* could be widely separated from oxidized cytochrome *c*. The difference in these two forms of the same protein is equivalent to one titratable group. In terms of charge, this is the smallest difference that can be achieved, and shows the fine degree of separation possible by this method. Most of the early work employing ion exchange resins for the chromatography of proteins (2) was confined to rather stable proteins with high isoelectric points. It was the work of Boardman and Partridge (3) which first demonstrated that the weak cation exchange resin, IRC-50, could be employed for the chromatographic separation of neutral, less stable compounds such as hemoglobin.

The procedure (4), as it was finally employed, was to adsorb the hemoglobin on a resin bed which was in equilibrium with a phosphate-citrate buffer, pH 6.3, and which had a sodium ion concentration of 65 mEq/l. The hemoglobin solution itself had been previously dialyzed against the same buffer. The protein was then eluted by gradually increasing the sodium ion concentration. The entire procedure was performed in a constant temperature room at 4°, since the hemoglobin molecule becomes denatured at more elevated temperatures.

Employing this technique, the red cell hemolyzate of the normal adult gave a chromatogram such as is shown in figure 1. Oxyhemoglobin is the form of protein employed, and can be detected by various colorimetric techniques. The protein is eluted in three fractions, one in the area of tube 10, the second at about tube 30 and the last at about tube 55. Spectrophotometric investigation of the three components showed that each had an identical oxyhemoglobin spectrum. If the compound is converted to the carbon monoxide derivative, the number and position of fractions remain constant.

To establish that the fractions were neither column artifacts nor conversion products of the hemoglobin molecule, the major component, that at tube 28, was rechromatographed. A single peak was obtained, as shown in figure 2, which demonstrated that only one compound was present. The same results were obtained on hemoglobin purified by starch block electrophoresis.

Our efforts then focused upon identification of the components. There have been a number of indications that hemoglobin A molecules dissociate. Field and O'Brien (5) for example, showed that between pH 3.5 and 6., a rapid reversible dissociation occurs into particles of about half the molecular weight. Other workers (6), employing free cell electrophoresis at low ionic strength, have been able to demonstrate multiple components in a preparation of hemoglobin A. These results had been interpreted as due to dissociation. Conclusions based on moving boundary electrophoresis indicated that the fragments of hemoglobin A have different charges. Earlier work by Moore and Reiner (7) showed that hemoglobin A, at low pH, is separable into two electrophoretic components with much lower sedimentation coefficients than the undissociated molecule. It was possible that this type of dissociation could account for the presence of the different hemoglobin components in the normal adult. If, for example, hemoglobin A dissociates into two halves, each slightly different in charge, then the three components could be accounted for by an equilibrium:

$$\begin{array}{ccc} & & \text{YY} \\ & & \downarrow\uparrow \\ \text{XY} & \rightleftharpoons & \text{X} + \text{Y} \\ & & \downarrow\uparrow \\ & & \text{XX} \end{array}$$

If this were the case, one might expect to find that

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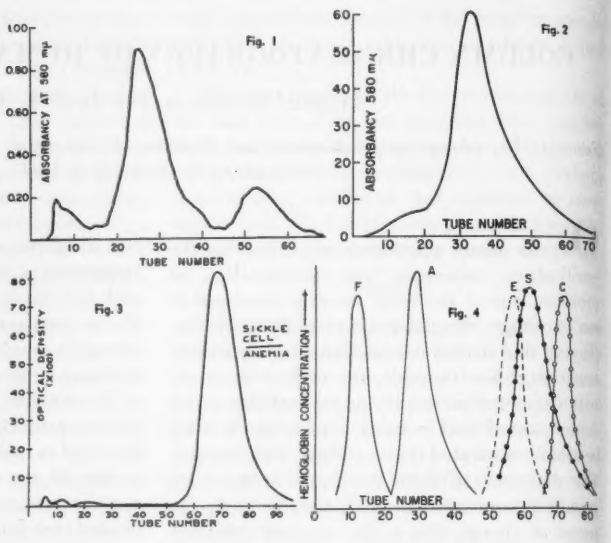

FIG. 1. Chromatograph of oxyhemoglobin obtained from red cells of normal adult.

FIG. 2. Chromatograph of purified hemoglobin A.

FIG. 3. Chromatograph of oxyhemoglobin obtained from red cell hemolyzate of individual with homozygous form of sickle-cell anemia.

FIG. 4. Composite chromatograph of hemoglobins F, A, E, S and C.

in the presence of high concentrations of urea, the reaction would be driven in the direction of the dissociated components and that the difference in charge would be great enough to allow separation of the components. However, when purified hemoglobin A was prepared in 4 molar urea and all the buffers employed for chromatography contained the usual salts plus the 4 molar urea, only a single component could be detected. It would appear that if the hemoglobin A molecule does dissociate under these conditions, the dissociated components could not be separated by our methods. Consequently, we felt that dissociation of hemoglobin did not account for the three hemoglobin components we observed in the normal adult.

Turning then to the chromatographic movement of other human hemoglobins, it was found that the hemoglobin of an individual which could be labeled by electrophoresis as homozygous hemoglobin S gave a chromatogram such as shown in figure 3. It was apparent that hemoglobin S moves as a homogeneous compound behind any of the components of the normal adult. This, then, actually demonstrated two things: first the column produced no multiple components with S hemoglobin, which further confirmed our previous data by suggesting that a single protein gives but a single peak in this method and second, that the genetic apparatus which gives rise to the multiple components in normal adult hemoglobin is completely altered in the homozygous sickle cell individual.

After a number of abnormal hemoglobins had been investigated, a composite chromatogram such as is shown in figure 4 could be drawn. Hemoglobin F (fetal) moves off the column in the region of tube 10, hemoglobin A at tube 30, hemoglobin E at tube 55, hemoglobin S at tube 65, and hemoglobin C at tube 75. Of the hemoglobins investigated, hemoglobin F moves off the column in the same position as the first fraction of normal adult hemoglobin, and hemoglobin E moves chromatographically as the third component of the normal adult.

There was substantial evidence in the literature that fetal hemoglobin did occur in the normal adult in small percentages (8). The most direct method of distinguishing fetal hemoglobin from adult or any of the abnormal hemoglobins is to assess its resistance to denaturation by alkali. After chromatography of the hemoglobin obtained from cord blood, the material which came off the column at tube 10 was resistant to alkali denaturation. This, as well as spectral analysis, definitely established it as fetal hemoglobin. However, examination of the component of the normal adult which came off at tube 10 indicated that it was not resistant to alkali denaturation. Further, on electrophoresis, the tube 10 component of adult moved more rapidly at pH 8.6 than hemoglobin A, while the fetal hemoglobin moves behind hemoglobin A at this pH. It would appear, then, that this first component contained in the red cells of normal adults was similar to that observed by Kunkel and Wallenius (9) by starch

block electrophoresis and Prins and Huisman (10) by column chromatography.

Turning to the third component of normal adult hemoglobin, it was noted that hemoglobin E moves off the column in the same position as this component. The electrophoretic movement of the third component was compared with that of hemoglobin E at pH 8.6 by paper electrophoresis. It was apparent from the results of chromatographic movement, as well as the results of electrophoretic movement, that this component of normal adult hemoglobin was at least closely related, if not identical to, hemoglobin E or the hemoglobin a_2 component of Kunkel (9, 11).

The results on the movement of the hemoglobins in column chromatography on the weak cation exchange resin are directly related to the charge carried by the protein. Relative mobilities are shown below.

On electrophoresis at

pH 8.6 A > F > S > E > C

pH 6.5 C > S > E > A > F

On chromatography at

pH 6.3 F > A > E > S > C

Below the isoelectric point, at pH 6.5, the hemoglobin will carry a positive charge. On electrophoresis below the isoelectric point, fetal hemoglobin has the lowest mobility, hence the smallest charge. Since it has the lowest positive charge, it is least strongly adsorbed on the column, and consequently moves off the column most readily (however, cf. ref. 12). Hemoglobin C, on the other hand, has the greatest electrophoretic mobility, hence it carries the greatest positive charge. Because it does carry this large charge, it is adsorbed most strongly to the column and is eluted last. Electrophoretic mobility and column chromatographic mobility are inversely related, and this relationship is a direct consequence of the charge carried by the protein.

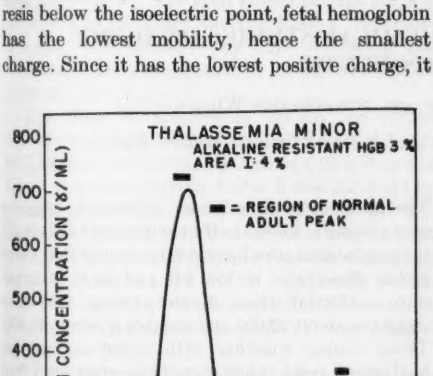

FIG. 5. Chromatograph of oxyhemoglobin obtained from red cell hemolyzate of individual with thalassemia minor.

In certain diseases, there appears to be a change in the distribution of the hemoglobin components found in the normal individual. Figure 5 shows the column chromatography of the hemoglobin obtained from individuals with the disease known as thalassemia minor. It is apparent that these individuals have small percentages of fetal hemoglobin and have a marked increase in the third component as well as an unidentified hemoglobin which moves off the column at tube 40. In the homozygous form of the disease, thalassemia major, there is a large percentage of fetal hemoglobin present but our third chromatographic component also makes up about the same percentage of the hemoglobin as it does in the individual with thalassemia minor. This contrasts with the results of the Rockefeller group (9) which reported that their a_2 component is present in individuals with the major form of the disease in about the same percentage as it is present in normal individuals.

Our efforts were also directed toward an evaluation of the chromatographic movement of oxyhemoglobin and methemoglobin. At a pH below the isoelectric point when a ferrous iron atom of oxyhemoglobin or carbon monoxyhemoglobin is oxidized to the ferric form, the protein will become more positively charged by about one titratable group. Thus, a molecule in which all four of the iron atoms are in the ferric form, methemoglobin, should be much more positively charged than a molecule in which all iron atoms are in the ferrous form. Boardman and Partridge (3) in their study, showed that they could completely separate bovine methemoglobin from bovine carbon monoxide compound. This was found to be true for human hemoglobins as well. Purified methemoglobin A will move off our columns much later than purified oxyhemoglobin A.

Direct attempts to isolate any intermediate forms, such as hemoglobin A which contains three iron atoms in the ferrous state and one iron

atom in the ferric state, had met with no success until recently. Then, Itano and Robinson (13) reported the separation of intermediate forms by moving boundary electrophoresis. When a hemoglobin solution was prepared in which the iron atoms were partially converted to ferric form, a number of fractions could be separated by chromatography. These fractions corresponded to hemoglobin in which the iron atoms were progressively oxidized. The movement of the different forms of hemoglobin A is shown below.

Compound	Carboxy Hb	Intermediates			Met Hb
Ratio Fe^{+2}/Fe^{+3}	4/0	3/1	2/2	1/3	0/4
Peak tube no.	28	54	65	75	90

As was the case with the abnormal hemoglobins, the forms of hemoglobin A move off the column according to the charge on the molecule. The molecule with all the iron atoms in the ferrous form is least positively charged and is eluted most readily, while the methemoglobin carries the highest positive charge and is the last form to be eluted. Thus, the forms of methemoglobin may lead to erroneous results in the quantitative analysis of mixtures of hemoglobin A and abnormal hemoglobins unless due care is exerted. However, the ability to separate the forms of methemoglobin should prove a valuable tool in a study of the kinetics of the individual steps of the oxygenation of hemoglobin.

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DISCUSSION: PHYSICAL AND CHEMICAL STUDIES ON HORSE GLOBIN COMPONENTS[1]

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The number and number of types of subunits in the molecule of horse hemoglobin has been a matter for debate. Porter and Sanger (1) estimated that there were six N-terminal valine residues by means of the fluorodinitrobenzene reaction and Ingram (2) obtained a similar result using phenylisothiocyanate. However, it has since been shown (3) that Porter and Sanger's method probably overestimated the number of end groups in human hemoglobin, casting doubt on their similar value for horse hemoglobin. It has long been known that horse hemoglobin tends to split in half under the influence of urea, mercaptoethanol and other reagents unlikely to affect covalent bonds (4, 5) The presence of four hemes, moreover, suggests four subunits. Recently Reichmann and Colvin (6) in these laboratories have demonstrated that horse globin dissociates at low pH and ionic strength into a material whose weight average molecular weight is about 22,000 and number average 16,500. These values, together with sedimentation behavior and some other properties, suggested four subunits of similar molecular weight 16,000. The higher weight average molecular weight was ascribed to incomplete dissociation.

The behavior of horse globin in this pH and ionic strength region has been explored further and it has been separated into two different proteins which are now being investigated. Since full details are being published elsewhere (7), only a brief synopsis will be offered here together with some very recent chemical characterization.

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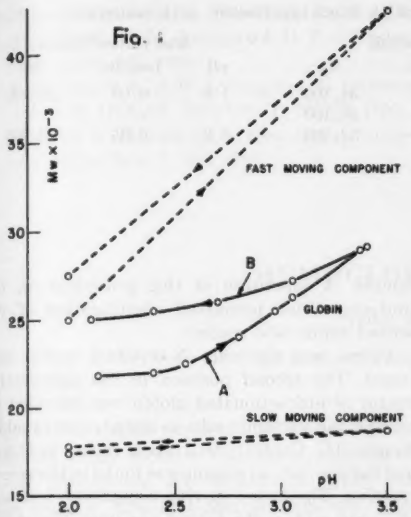

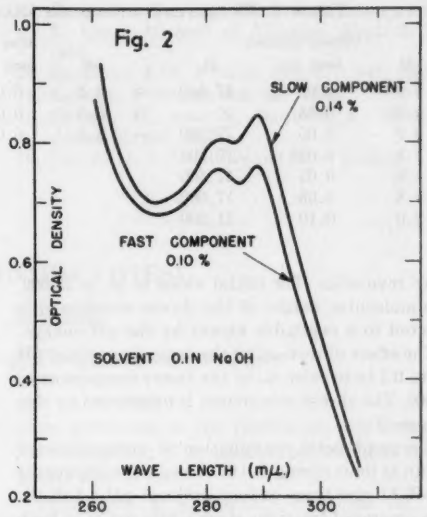

FIG. 1. Weight average molecular weights of globin and globin components in ionic strength 0.05 NaCl-HCl as a function of pH.

FIG. 2. Ultraviolet absorption spectra of globin components.

The globin used in this work was prepared from twice recrystallized oxyhemoglobin obtained by the method of Heidelberger (8). The heme was removed by three precipitations at −15°C with acid acetone following the procedures of Jope, Jope and O'Brien (9). The final globin precipitate was dissolved in ice-water and lyophilized. Molecular weights were measured by light scattering and confirmed by sedimentation, using Archibald's method (7).

The effect of changes in pH on the weight average molecular weight (M_w) of globin in NaCl-HCl medium at an ionic strength of 0.05 is shown in figure 1, central portion. *Curve A* was obtained by adding alkali to a solution initially at pH 2.1; *curve B* by adding acid to a solution originally at pH 3.4. These results indicate that the changes in M_w on altering the pH are not completely reversible. The same partial irreversibility was found in a series of experiments where the pH was changed by dialysis (7).

Table 1 shows the effect of altering the ionic strength on the molecular weight of the protein at a given pH. Increasing the ionic strength at pH 2.0 from 0.05 to 0.1 NaCl-HCl causes an increase in molecular weight which is not reversed by dialysis back to the lower ionic strength. The further increase in molecular weight between 0.1 and 0.2 is largely reversible. At pH 3.3, the molecular weight is the same at ionic strengths 0.05 and 0.1, but further increase causes extensive aggregation.

We have recently found that globin is dissociated to a greater extent in formate buffers at about pH 2 than by chloride media. Table 2 shows the weight average molecular weights in initial solution and after dialysis. As in chloride media, the aggregation on raising the pH is reversible between pH 3.4 and 5, but only partly reversible between pH 1.8 and 3.4. At pH 2 and below, the molecular weight is about 17,000 at an ionic strength below 0.05 but increases if the latter is raised to 0.1.

TABLE 1. MOLECULAR WEIGHT OF GLOBIN IN CHLORIDE MEDIUM

Original Solvent			Solvent After Dialysis		
pH	Ionic str.	M_w	pH	Ionic str.	M_w
2.0	0.05	21,500			
2.0	0.10	27,500	→ 2.0	0.05	29,100
2.0	0.20	49,300	→ 2.0	0.05	33,800
3.3	0.05	29,500			
3.3	0.10	29,400			
3.3	0.20	78,000			

Under those conditions where the molecular weight approaches its lowest value, (i.e. ionic strength 0.05 and pH below 2) the electrophoretic pattern shows two components (6, 7). By means of Kekwick's modification (10) of the Tiselius cell, samples of the faster moving component were obtained from the ascending limb and of the slower component from the descending limb.

The effect on the isolated components of altering the pH at ionic strength 0.05 NaCl-HCl media is shown in figure 1. The faster component aggregates readily as the pH is raised and the effect is not

TABLE 2. MOLECULAR WEIGHT OF GLOBIN IN FORMATE-FORMIC ACID MEDIUM

Original Solution			After Dialysis			After Further Dialysis		
pH	Ionic str.	M_w	pH	Ionic str.	M_w	pH	Ionic str.	M_w
1.8	0.05	17,500 →	3.5	0.05	34,100 →	1.9	0.05	22,300
1.8	0.05	→	5.0	0.05	53,100			
4.9	0.05	77,200 →	3.4	0.05	32,500 →	1.9	0.05	22,200
1.8	0.025	17,200						
1.8	0.05	17,500						
1.8	0.05	17,000						
2.0	0.10	31,200						

quite reversible. The initial value of M_w is 25,000. The molecular weight of the slower component is affected to a negligible extent by the pH change.

The effect of increasing the ionic strength at pH 2.0 to 0.1 is to raise M_w of the faster component to 33,000. The slower component is unaffected by this change (7).

Electrophoretic examination of unfractionated globin at ionic strength 0.05 shows two components at pH 2.0 and three or four peaks at pH 3.5 (6, 7). The separated fractions show single peaks at both pH values (7). The extra peaks appearing in the electrophoretic pattern above pH 2.0 are probably due to aggregates involving both components. Aggregates of the faster component with itself do not differ electrokinetically from the monomer.

Chemical differences may also be demonstrated between these two materials. Figure 2 shows their ultraviolet absorption spectra. The difficulty in locating the base line precludes accurate determinations of the tyrosine and tryptophane contents but the shape of the curve indicates that the tyrosine-tryptophane ratio is lower in the fast moving component (11).

The N-terminal amino acids of globin and its two components have been investigated by the Edman phenyl isothiocyanate method (12). The first stage of the reaction was complete in one hour at pH 9.0 in 50% pyridine at 40°. The reaction mixture was extracted with benzene to remove pyridine, and the phenyl thiocarbamyl globin was precipitated with acetone and ether and washed with ether. The precipitate was dissolved in a mixture of glacial acetic acid and constant boiling HCl (2:1), and after 30 min. at room temperature, a derivative of the N-terminal amino acid had split off. The residual protein was then removed from the solution by precipitation with acetone and ether. The derivative of the N-terminal amino acid, after evaporation of the acetone and ether, was converted to the phenyl thiohydantoin (PTH) by treatment with 3N HCl at 40° for 1 hour. The PTH was extracted with ether and the amount estimated from the ultraviolet absorption (12) of the ethereal extract. Paper chromatography with Sjoquist's (13) solvents A and C was used to identify the PTH by comparison with an authentic sample. A repetition of this procedure on the protein residue permitted identification of the second amino acid residue.

Valine was the only N-terminal amino acid found. The second position in the polypeptide chains of unfractionated globin was occupied by leucine and glutamic acid as already indicated by Sanger (14). Contrary to a recent report by Ozawa and Satake (15), no glycine was found in the second position. The faster moving component had only glutamic acid in the second position. The slower component had mainly leucine in the second position. A trace of glutamic acid is ascribed to contamination with the fast component since the spreading boundaries in the descending limb of the electrophoresis cell make a clear cut separation difficult.

The four subunits into which horse globin (molecular weight about 64,000) can be dissociated by suitable adjustment of pH and ionic strength seem, therefore, to be of two types. One of these types has an N-terminal sequence of valyl-glutamyl-, or perhaps of valyl-glutaminyl-, and the other of valyl-leucyl-. The two types appear to be present in equal amounts (16).

The molecular weights of both materials are similar, about 16,000, but one aggregates readily as the pH and ionic strength are raised from 2.0 and 0.05 respectively, while the other is unaffected. Both, after oxidation with performic acid and hydrolysis, give rise to one residue of cysteic acid per mole (16) but they differ in tyrosine and tryptophane content.

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HEMOGLOBINOPATHIES[1]

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Apart from their physical and chemical properties the many recently discovered variants of human hemoglobin are of interest chiefly for three reasons. The first and perhaps the most important one is the fact that the molecular structure of hemoglobin is under genetic control. The second is the significance which the composition of hemoglobin in a given individual has for the physiology and pathophysiology of the carrier. The third is connected with the strikingly uneven distribution of the various types of hemoglobin in different populations and the meaning of this fact in terms of anthropology and human ecology.

As is well known, the fetus elaborates an alkali-resistant hemoglobin, called hemoglobin F. This hemoglobin is produced regardless of the ultimate pattern of 'adult' hemoglobin and must be regarded as normal, although it may be identical with alkali-resistant hemoglobin which is found under pathologic conditions in later life as well as with the trace amounts of alkali-resistant hemoglobin of normal adults. The production of hemoglobin F appears to be subject to special as yet obscure, genetic mechanisms. Most of the now known adult hemoglobins are under the control of genes which, ordinarily at least, behave like simple Mendelian dominants. The normal individual produces in extrauterine life a hemoglobin which, except for the recently confirmed minor components, is essentially homogeneous and corresponds to the traditional genetic formula AA. The gene responsible for the production of hemoglobin S, when present in single dose, gives rise to the phenotype AS, the gene for hemoglobin C to phenotype AC, that for G to AG and so on.

Penetrance is apparently complete for the sickling gene and the hemoglobin C gene and is likely to be high for several other 'hemoglobin genes,' although the data are as yet incomplete. The combination of an abnormal gene with its normal allele is thus usually expressed by the production of both the corresponding types of adult hemoglobin, A as well as the abnormal variant. Under ordinary circumstances more than 50% of these natural mixtures is hemoglobin A.

Homozygosity for the variant gene implies the absence of a normal allele necessary for the production of hemoglobin A. In such individuals no hemoglobin A is actually found though hemoglobin F may be present in significant amounts. The only adult type hemoglobin produced by the homozygote is that corresponding to the abnormal gene, the phenotype is S or C or G or E, as the case may be, and the corresponding genotypes are SS, CC, GG or EE.

In these examples phenotypes and genotypes happen to coincide, but this is by no means always the case. Homozygosity for a gene which leads to the formation of an abnormal component does produce a homogeneous hemoglobin, but the reverse is not always true. Physical-chemical homogeneity of hemoglobin is not necessarily the expression of homozygosity for the corresponding gene.

The homogeneity of a given sample may, of course, be only apparent and result from technical limitations inherent in the method of analysis. Thus paper electrophoresis does not sufficiently

[1] Supported by a grant from The Rockefeller Foundation.

discriminate between S and D, S and G, or D and G, or at pH 8.5 between H and I, when further analysis of such mixtures by a variety of other methods would disclose the heterogeneity. But such technical aspects apart, even a truly homogeneous hemoglobin present as a single component does not necessarily indicate homozygosity for the gene which determines its presence.

The reason appears to lie in the existence of one or possibly several unrelated genes which are not themselves capable of producing major qualitative changes in the structure of adult hemoglobins but can 'interact' with the hemoglobin genes. Such a gene is the thalassemia gene, which because of its interaction effects is of profound importance to any consideration of the abnormal hemoglobins. The thalassemia gene, in an otherwise normal individual, appears to interfere in a quantitative rather than qualitative manner with the production of hemoglobin A. Heterozygotes for this gene have erythrocytes which contain somewhat reduced concentration of A, while homozygotes exhibit severe hypochromasia of their erythrocytes and much of the hemoglobin they are able to produce is not A but F.

Interactions between a thalassemia gene and a single hemoglobin gene may greatly alter the phenotype expected in a heterozygote and actually simulate homozygosity with respect to the gene for an abnormal hemoglobin. In such situations the formation of A may be reduced or altogether suppressed while the proportion of the abnormal hemoglobin is correspondingly increased. Cases in which double heterozygosity for thalassemia and an abnormal hemoglobin trait have simulated homozygosity for the latter by the complete absence of demonstrable hemoglobin A have been described in sickle cell thalassemia disease. Recently we found a similar situation in the combination hemoglobin G-thalassemia in which only hemoglobin G was demonstrable. Obviously, then, genetic data must supplement the hemoglobin analysis before a genetic diagnosis can be attempted.

The significance of the small amount of hemoglobin A often found in the double heterozygotes of sickle-cell thalassemia deserves reinvestigation in the light of the recent demonstration that A itself is not homogeneous, but contains several minor components demonstrable by starch block electrophoresis and other methods. As yet little is known about the genetic control of these minor components. It is entirely conceivable that certain genes and gene combinations suppress the production of the major component of A while permitting the formation of minor components, such as the so-called A_2, which are perhaps regulated by other genes.

That this may well be the case is suggested by findings we have recently made in another type of double heterozygote in which according to previous genetic theory no A at all should be present. I am referring to the combination of the sickling gene and the hemoglobin C gene. Considerable evidence suggests that these genes are alleles of one another, of the gene for D and, of course, of a gene involved in the formation of hemoglobin A. If a single gene regulating the production of the entire A complex were part of a series of multiple alleles, then the combination SC should not show any hemoglobin A, and this has hitherto been assumed to be true. Using a method developed in our laboratory by Robinson and his associates (1) which involves a combination of electrophoresis and absorption on an agar plate buffered at an acid pH, we were able to show the presence of a small component in the A position in an individual whose major hemoglobins and genetic background indicated the genotype SC. The method likewise demonstrated a small A-like component in an individual presumed to be homozygous for S. We do not as yet know whether this component is identical with that of Kunkel's A_2 on starch block electrophoresis. The agar method has enabled us to demonstrate small amounts of F, to separate F from G, and to characterize differences between S and D, components which formerly could only be distinguished by their solubilities. Under the conditions chosen by us, the combination AD shows a single spot on the agar plate, while S and G separate from A. Each additional method of characterization increases the complexities of the genetic analysis.

The allelic relationship of the various hemoglobin genes is still for the most part obscure. Reference has been made to the probable allelism of the genes for S, C and D. Several observations indicate the thalassemia gene—or genes, for it is likely that more than one gene with similar effects on hemoglobin metabolism may exist—the thalassemia gene is non-allelic to the A-S-C-D group. Transmittance of both the S trait and thalassemia from one parent has been demonstrated. A recently published detailed analysis

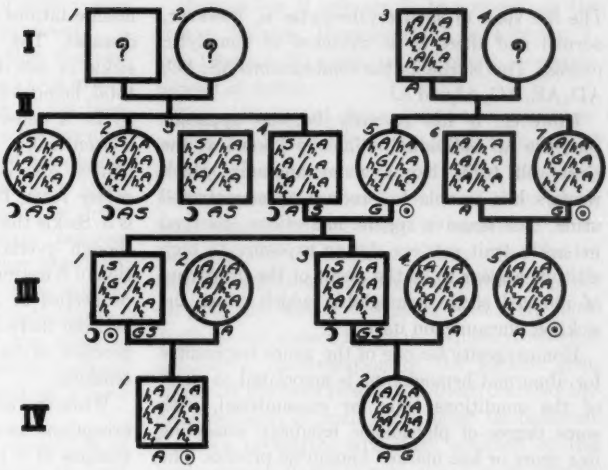

FIG. 1. Pedigree of family with combinations of hemoglobin G, hemoglobin S and thalassemia. Symbols within circles and squares represent the assumed genotypes on the basis of 3 separate loci. Letters and symbols on the outside indicate the phenotypes.

of a family in which, by a fortunate coincidence there was commingling of three abnormal genes, G, S and thalassemia (2), has shown that the gene for hemoglobin G cannot be allelic to either the gene for S and its alleles or to the thalassemia gene.

Already these three separate loci for genes concerned with hemoglobin structure have been demonstrated and it is to be expected that more will follow. It is clear that the relatively simple original assumptions regarding the genetics of hemoglobin will not suffice, and Neel has proposed a new terminology to accomodate the now established facts and allow for future discoveries in this field. We have used this approach in constructing a pedigree of the family with the S, G and thalassemia abnormalities, which indicates the complexity required to do justice to the facts. For each known locus a pair of genes is assumed and a corresponding notation is assigned, h_1 for the first known locus, the S-C locus, h_2 for the G locus and h_t for thalassemia. It is of interest that III-3 in this pedigree (fig. 1), heterozygous for the non-allelic genes S and G had no demonstrable A. The phenotype was GS. The phenotype of II-5 was G. The production of the so-called normal hemoglobin, A, in the light of such findings cannot be dependent on a single gene but appears to depend on a favorable constellation of several genetic factors. Conversely, the absence of A hemoglobin in the phenotype is not in itself evidence for the allelism of two abnormal genes combining in a double heterozygote.

The physiologic consequences of the presence of abnormal hemoglobins are no less intriguing than the genetic aspects. Before attempting to discuss the various syndromes and hematologic manifestations it may be well to pause for a brief reminder that the very term 'hemoglobinopathies' which has gained general acceptance and forms the prescribed title of this presentation, reflects a specific bias, the bias introduced by the method of ascertainment. Most of the abnormal hemoglobins have come to light through clinical investigation of hematologic states. Their presence is not necessarily a physiologic handicap, and even in frankly abnormal conditions the existence of a hemoglobin other than A may be coincidental and not contributing to the disturbance at hand. Such appears to be the case with respect to hemoglobin G, and may well be true for other variants. The term hemoglobinopathy denotes pathologic significance, whereas it is entirely conceivable that at least some hemoglobins should be looked upon as merely normal variants like the blood groups, and even that their presence in certain environments may actually confer an advantage rather than a disadvantage upon the carrier.

The weakness of the term becomes all the more obvious when it is realized that, so far, without exception the simple heterozygous states in which the abnormal hemoglobin exists in combination with hemoglobin A, appear to be, under ordinary circumstances at least, benign asymptomatic conditions manifested only by the physico-chemical pattern and, in some instances by shape anomalies of the erythrocytes.

The life span of such erythrocytes is, however, normal and there is no evidence of hemolytic process. This is true for the combinations AS, AC, AD, AE, AG, AI and AJ.

However, it has recently become apparent that the AS individual, clinically known as the sickle-cell trait, has a renal anomaly which renders him unable to produce a concentrated urine. The massive splenic infarctions observed in sickle trait carriers during exposure to high altitude appear to be the result of the properties of reduced sickle hemoglobin which cause the sickling phenomenon itself.

Homozygosity for one of the genes responsible for abnormal hemoglobins is associated in most of the conditions thus far encountered, with some degree of physiologic handicap consisting in a more or less marked hemolytic process. The same is true for the states of double heterozygosity involving the presence of two abnormal hemoglobin genes, or of one such gene in combination with a thalassemia gene. The life span of the erythrocytes of such individuals is usually shortened, there is a compensatory erythroid hyperplasia of the bone marrow and, in many instances, a marked degree of splenomegaly which suggests a relationship between the structure of the erythrocytes which contain the abnormal mixture, and splenic mechanisms. Usually there are also shape anomalies of the erythrocytes.

The best studied and relatively best understood combinations are those involving the sickling gene and the presence of sickle-cell hemoglobin or hemoglobin S. Five such combinations have been studied in addition to the already mentioned simple heterozygote AS. They are the homozygous state, clinically designated as sickle-cell anemia, phenotype S or SF; sickle-cell-thalassemia, i.e. double heterozygosity for the sickle gene and a thalassemia gene, phenotype SAF or SF; sickle-C disease, the combination of the genes for S and C, phenotype SC; sickle-cell hemoglobin D disease, genotype and phenotype SD; and the combination SG which behaves remarkably like a simple AS trait.

The greatly decreased solubility of reduced S hemoglobin is the feature which sets this compound apart from all other types thus far encountered, and is the reason for the peculiar shape distortion of the erythrocytes known as sickling. *In vivo* sickling is regarded as the basis of vascular occlusion and of numerous clinical manifestations occurring in the various sickle-cell diseases. The solubility and the readiness to sickle *in vivo* depend on the proportions of the total hemoglobin mixture which is represented by S. A general correlation exists between the concentration of S in a given syndrome and clinical severity. Sickle-cell anemia is the most severe form, because nearly all the hemoglobin is S. Sickle thalassemia is somewhat less severe—though overlapping occurs—with a concentration of S nearer 70%. SC and SD are milder, with properties of 50% S, and the trait is benign because there is less than 50% S, not enough to produce *in vivo* sickling under ordinary oxygen tensions.

While this scheme has some validity, important exceptions have been described in which concentrations of S in the range of 70–100% were not associated with the hematologic or physiologic disturbances usually present. Some of these cases may have been sickle-thalassemia disease in which the thalassemia gene was not interacting in the usual manner, others may be true homozygotes for the S gene, still others as yet unrecognized genetic combinations. The point is, regardless of genetic background, the concentration of S in the hemoglobin mixture is not the sole determinant of the physiologic effects of the sickle gene. Additional and as yet obscure factors must be postulated. These factors may reside in stromal structure, or plasma components or in still other physiologic mechanisms on which we can only speculate at present. In any event caution is necessary in correlating clinical-physiologic phenomena with molecular structure of hemoglobin.

Our present state of ignorance is even more profound with respect to the physiologic significance of the other components. It is of interest that the homozygous states for three distinctly different hemoglobinopathies, C, D and E are associated with leptocytosis, i.e. the presence of target cells. The combination of C and E with thalassemia have a remarkable clinical and hematologic resemblance.

The known facts about the structure and solubility of the components C, D and E give no clue as to why their presence should be associated with shape anomalies or hemolytic anemia. For the more recently described hemoglobins I, J and K, only combinations with A have thus far been found. H has only been recognized in combination with a thalassemia-like gene and

may indeed require the presence of such a gene in order to become phenotypically apparent.

Hemoglobin G is of interest in that thus far no pathologic state could be assigned to it in any of the four combinations in which it has been encountered. AG as well as the only documented case of homozygous G have appeared hematologically normal. GS behaves like a sickle trait. G-thalassemia does not seem to differ from thalassemia minor. G-S-thalassemia behaves like S-thalassemia. It would appear therefore, that hemoglobin G may merely be a normal variant of A, of equal value to the individual as the commoner A itself. Whether the same is true of the other, newer hemoglobin variants will have to await the development of a new body of data.

One cannot leave the subject of variability of human hemoglobins without making at least mention of the distribution of the various hemoglobins in different parts of the globe. Thalassemia or Mediterranean anemia derives its very name from the fact that the gene giving rise to this condition is concentrated around the Mediterranean basin. We know now, however, that thalassemia or thalassemia-like states are common in other parts of the world.

The sickling gene has a distribution which shows maximal incidence in Africa south of the Sahara, but with marked variations from tribe to tribe, reaching about 40% incidence in some populations. It has also been encountered in India, at various points intermediate between India and Africa and in rather sharply localized areas of Greece, Sicily and other parts around the Mediterranean.

Hemoglobin C has its highest incidence in West Africa with a rather sharp peak around the Gold Coast and a fairly steep gradient. No hemoglobin C has been found in East or South Africa.

Hemoglobin E is very prevalent in the Orient, India, Indochina and Indonesia.

Hemoglobin H appears to be common in the Orient likewise. Hemoglobin J or Liberian I, and Liberian II, again are prevalent in certain regions of Africa.

The meaning of these patterns for anthropology is obvious. Their meaning in terms of ecology poses some very provocative questions. If it is considered that for most of these genes some degree of handicap is associated with homozygosity, it becomes clear that under natural conditions homozygotes are less apt to survive. The loss of two genes with every such death should reduce the gene frequency, whereas actually a concentration of the variant gene occurs. It has been postulated, therefore, that the disadvantages of such genes must be balanced by some protective advantage confined on the heterozygotes in relation to one or more specific environmental factors. For the sickling gene it is widely thought that this balanced polymorphism consists in relative protection of the sickle trait against falciporous malaria. Presumably sickle hemoglobin offers a less favorable environment to the parasite than does normal hemoglobin. The evidence for this hypothesis is still incomplete.

What factors contribute to the concentrations of the genes for C, E, thalassemia, or the newer hemoglobins is unknown. Here then is a fruitful field for investigation in which the disciplines of biochemistry, hematology and clinical medicine join the anthropologist, the geneticist, and perhaps workers in nutrition, parasitology and entomology, public health and many others.

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AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

Symposium on Structure and Function of Proteins[1]

Chairman: HANS NEURATH

INTRODUCTION

HANS NEURATH

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THE present symposium has been arranged for the purpose of bringing to focus our current state of knowledge of the relation between the biological specificity and the chemical structure of proteins. Such an undertaking may appear bold and premature to the critical observer who expects a quantitative formulation of the biological function of a protein in molecular terms, particularly if one aims his sight at the ultimate objective of predicting the biological properties of a protein from its molecular configuration. On the other hand, tremendous advances have been made in determining the chemical structure and structural characteristics of proteins of specific biological functions, and there is good reason to hope that within the foreseeable future we shall be able to pinpoint the minimum structural characteristics required for specific biological functions of certain proteins. It is against the background of this objective that the program of this symposium has been arranged.

The current period of protein research derives its impetus from several fundamental methods and concepts of recent origin. These include, first of all, refined methods for the separation and isolation of proteins, peptides and amino acids, largely by methods of chromatography and electrophoresis in immobilized media. We have rediscovered the usefulness of proteolytic enzymes in the controlled degradation of proteins; and the complete elucidation of the chemical structure of insulin, in the hands of Fred Sanger (1), may be regarded as a triumphant demonstration of the simplicity, ingenuity and resolving power of these methods of analysis. In the succeeding years, we have quickly accepted as a matter of course the elucidation of the amino acid sequence of other proteins, and our inquiries have become directed toward the secondary and tertiary structure of proteins involving hydrogen bonding and various types of side-chain interactions which cause the polypeptide chain to follow or depart from helical patterns (2, 3). The method of optical rotation has come to the fore as a valuable tool in assessing the internal configuration of proteins and the changes occurring in various types of reactions of biological interests (4, 5); and there is a feeling of expectation that a clue to the biological specificity of proteins will be found in a unique structural pattern of an amino acid sequence of a discrete region of the protein molecule.

Today we are less certain than ever how small or large a structure has to be in order to constitute a protein; and as we try to interpret the biological function of a protein in terms of a discrete spatial arrangement, peptides and peptide fragments assume greater importance. It is fitting, in this context, to call attention to the outstanding achievements of du Vigneaud and his co-workers who have elucidated and synthesized the structure of the peptides, oxytocin and vasopressin (6), and who have accomplished the synthesis of oxypressin (7), a hybrid structure containing the moieties required for the physiological activity of each of the two hormones, and combined in a single molecule. Other compounds in the twilight zone between peptides and proteins, whose chemical structure has been fully elucidated, include the cortico-

tropins (8), the melanophore-stimulating hormone (9), glucagon (10), and several others, and it is unfortunate that time does not permit their consideration as part of this symposium.

Included in the discussion this afternoon are first, a group of protein hormones obtained from different zoological species; then ribonuclease, an enzyme which has been the subject of a painstaking inquiry by Drs. Moore and Stein, as reported at last year's Federation meetings (11), and by Dr. Anfinsen, who will appear before us today. The efforts directed toward the stepwise degradation of a proteolytic enzyme without impairing its catalytic properties will be documented by Dr. Emil Smith's work on crystalline papain; the structural changes involved in the formation of proteolytic enzymes from their inactive precursors will be the subject of a paper by Dr. Dixon and myself. Our inquiries will be concluded by a discussion by Dr. Fraenkel-Conrat of the structural aspects of one of the largest known macromolecules, namely, tobacco mosaic virus. And while I do not wish to anticipate your reactions to what each of the speakers will have to say, I am rather confident that you will emerge from this session with a feeling of excitement about the accomplishments to date and those which they foreshadow for the foreseeable future.

This symposium was intended to be primarily informative and, hence, discussion time had to be sacrificed in order to accommodate the following five papers within an afternoon session. Fortunately, other protein symposia have been scheduled this year for the purpose of exchanging information, including the Gordon Conference in early July, and the Paris Conference later in the same month.

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PROPERTIES OF AND STRUCTURAL INVESTIGATIONS ON GROWTH HORMONES ISOLATED FROM BOVINE, MONKEY AND HUMAN PITUITARY GLANDS[1]

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The relation of the pituitary gland to growth was first given experimental demonstration by Cushing and his collaborators (1) in their classical experiments on the hypophysectomy of dogs in 1910, and by 1930 investigations with various species, including fish, amphibia, reptiles, birds and mammals, had left little doubt that the anterior pituitary contains a hormone whereby

[1] The original experiments reported here were aided in part by grants from the American Cancer Society and from the Albert and Mary Lasker Foundation.

resumption of growth is induced in hypophysectomized animals. During the period between 1944 and 1948, two groups of investigators (2, 3) have described methods for the isolation of growth hormone[2] in a highly purified form from the anterior lobes of beef pituitaries. During the past few years, many attempts (7–10) have been made to purify further the products isolated by the published procedures (2, 3). The failure of these attempts has lent support to the belief that the protein is the hormone (11).

The bovine somatotropin is a protein with a molecular weight of 45,000 and an isoelectric point of pH 6.85; the protein nitrogen can be accounted for completely by its amino acid and amide content (11). The hormone protein consists of a branched polypeptide chain with two N-terminal residues (phenylalanine and alanine) and only one C-terminal residue (phenylalanine), and a total of 396 amino acids (fig. 1). The N-terminal residues (12, 13) were determined by means of both the fluorodinitrobenzene (FDNB) and phenylisothiocyanate procedures, and the N-terminal amino acid sequences (14) were established by the isolation and identification of DNP-peptides from partial acid hydrolysates of DNP-somatotropin. Reaction of the hormone protein with lithium aluminum hydride (15) and hydrazine provided the evidence for the existence of on y one C-terminal phenylalanine. The C-terminal amino acid sequence was elucidated by submitting the hormone to the action of carboxypeptidase (16). According to the proposed structure for the bovine somatotropin, as

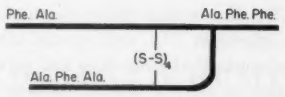


FIG. 1. Proposed structure for bovine growth hormone.

shown in figure 1, cleavage of the —S—S— bridges should not give rise to two fragments because the branched chain is probably derived from a peptide linkage involving the ϵ-amino group of a lysine residue. Studies of the product obtained by the performic-acid oxidation of the hormone protein (14) were in agreement with the proposed structure. It may be further noted that the proposed structure for the bovine hormone requires the formation of a peptide bond whereby the ϵ-NH_2 group of a lysine residue would be joined with the α-COOH group of another amino acid residue; with respect to the cystine residues in this structure, some of them may form intermolecular —S—S— bridges along the chain, instead of cross-links between the chains.

When the bovine growth hormone from which two residues of phenylalanine had been removed by carboxypeptidase was assayed for biological activity, it was found according to results of both tibia-width and body-weight assay that C-terminal phenylalanine is not essential for the biological function of the hormone (16). With an enzyme/hormone ratio of 1/300 at a temperature of 25° in a buffer of pH 9.5, the rate of hydrolysis of the bovine hormone by chymotrypsin follows a zero-order reaction up to 4 hr. of digestion (17). It was found that chymotryptic hydrolysis to the extent of about 25% did not cause inactivation, but that longer digestion did abolish the biological potency. It is worthy of note that if an active digest is desired, the degree of hydrolysis must not be permitted to exceed 30%. The non-protein nitrogen can be separated from the whole digest either by dialysis or by treatment with a 5% solution of trichloroacetic acid. It was demonstrated by biological assay that the growth-promoting activity resides in the remaining components (the core), which are non-dialyzable and insoluble in the trichloroacetic acid solution (17). When the results of a multiple-dose assay of the core are compared with those of a similar assay of the undigested hormone (table 1), statistical analysis of the data showed that there is no significant difference between the core and the

[2] The term 'growth hormone' has recently led to much confusion which is chiefly derived from the difficulties of defining the word 'growth'. In 1954 at the International Symposium on the Hypophyseal Growth Hormone, Weiss said: "Since growth processes are so diverse and composite, it would be a miracle if any one hormone were to act on all of them in such fashion as to always net a positive balance, which would make it a veritable 'growth hormone'. Any hormone affecting any one of the innumerable component steps of growth, is in a sense a 'growth hormone', although nothing seems to be gained by naming it so." (4).

For many years, the pituitary growth hormone was thought to possess only general body growth-promoting activity, as its name indicates, and any observed biological effects other than the promotion of growth were attributed to some contaminating factor or factors. Since so many effects involving various tissues or organs have now been ascribed to the pituitary growth hormone (5), it has been proposed to designate the hormone *somatotropin* (6).

untreated material with respect to growth-promoting activity.

Boundary electrophoresis of the core has given no indication of the existence of a component which behaves like the untreated bovine hormone. Analyses of the N-terminal residues of the core reveal a number of new residues (threonine, serine, tyrosine, lysine, etc.), in addition to phenylalanine and alanine; if we assume that terminal phenylalanine and alanine residues have come from the undigested bovine hormone, it can be estimated that the native hormone in the core amounts to less than 20%, a percentage certainly not sufficient to account for the biological activity of the core. From these results, it may be concluded that the activity does not depend upon the integrity of the bovine protein, and that the growth-promoting activity resides in a center (or centers) of activity in the molecule.

Table 1. Growth promoting activity of core from partial chymotryptic digest of bovine somatotropin

Preparation	Total Dose μg	No. Rats	Tibia Width* μ
Untreated hormone	20	8	207 ± 5.1
	60	8	239 ± 3.6
	200	8	271 ± 4.4
Core	20	8	202 ± 3.5
	60	7	232 ± 3.1
	200	7	277 ± 5.5

* Mean ± standard error.

Soon after the demonstration of corticotropins (ACTH) as an effective therapeutic agent in clinical applications (18), many attempts have been made to find the effectiveness of the bovine growth hormone in man, but they have met with disappointment. One of the obvious explanations for this failure is that the bovine growth hormone is chemically different from the hormone derived from human glands. Indeed it has been shown that the growth hormone prepared from fish pituitaries is active in fish (19), but not in rats (20); and that monkeys are not responsive to bovine growth hormone, whereas they are

Table 2. Protocol for isolation of human growth hormone

Fraction	Wt., mg	Procedure	Growth-Promoting Activity,* μ	N-terminal Residues†				
				Phe	Glu-Asp	Ala	Tyr	Val
A	1000	Lyophilized whole pituitaries	206	0.39	0.16	0.09	0.17	0.19
AS	530	Extracted with pH 10.5 CaO solution; fractionated with $(NH_4)_2SO_4$ ↓						
B	210	1.9 M $(NH_4)_2SO_4$ precipitate, dialysed and lyophilized ↓ Extracted with 0.45 M $(NH_4)_2SO_4$ in pH 5.1 phosphate buffer ↓	213	0.53	0.14	0.09	0.10	0.14
C	140	Soluble fraction submitted to chromatography on IRC-50 resin column ↓	221	0.65	0.12	0	0.11	0.12
D	75	Active peak; recovered by $(NH_4)_2SO_4$ precipitation as B ↓ pH and ethanol fractionation ↓	235	0.87	0.13	0	0	0
E‡	40	5–20% (v/v) ethanol precipitate; dissolved and lyophilized	248	1.00	0	0	0	0

* Assayed at a total dose of 20 μg in 4 days by the tibia test; 6 hypophysectomized rats in each assay.
† DNP-amino acid/total DNP-amino acids. ‡ Growth hormone.

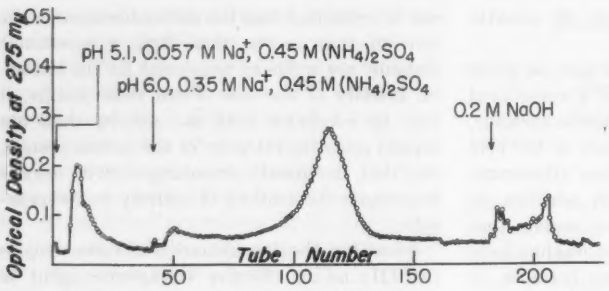


FIG. 2. Chromatography on Na^+ form of amberlite XE-97 resin (dimension of column, 3 by 30 cm) of a growth hormone concentrate (100 mg) obtained from human pituitaries; 10 ml per tube. The hormone activity is located in tubes 99 to 127.

responsive to growth hormone prepared from pituitaries of their own species (21, 22). Our recent investigations with human and monkey pituitary glands indicate that the human and monkey growth hormones are similar in structure and properties, but that they both differ completely from the hormone molecule isolated from the pituitaries of cattle (23).

Isolation of Human and Monkey Somatotropins. Table 2 outlines the procedure for the isolation of growth hormone from either monkey or human pituitary glands. Briefly, 1 gm of lyophilized human pituitaries[3] was twice extracted with 50 ml of a CaO solution of pH 10.5, and an active concentrate was then obtained by precipitation with 1.9 M $(NH_4)_2SO_4$, as described for the preparation of the bovine growth hormone (24). The $(NH_4)_2SO_4$ precipitate (0.21 gm) was extracted three times with 35 ml of a phosphate buffer of pH 5.1, containing 0.057 M Na^+ and 0.45 M $(NH_4)_2SO_4$. The clear extract was submitted to chromatography on the polycarboxylic acid resin Amberlite IRC-50 (XE-97) under the conditions shown in figure 2. The contents of tubes 99–127 were combined and the active component was obtained by the addition of an equal volume of saturated $(NH_4)_2SO_4$ solution. The resulting precipitate (0.075 gm) was dissolved to make a 50-ml solution and dialyzed. The dialyzed solution was brought first to pH 4.5 and then to pH 5.5; any precipitates formed at these two pH's were removed by centrifugation. The clear supernatant fluid was then diluted to a 0.2% solution; 40% ethanol (volume for volume) was added slowly with vigorous stirring until the concentration of ethanol reached 5% by volume. The precipitate formed was removed by centrifugation and discarded. The supernatant fluid was brought to 20% ethanol; ethanol fractionation was performed at 0° C. The 5–20% ethanol precip tate was dissolved in a solution of pH 7 and lyophilized. The yield was 40 mg of a product which behaves as a homogeneous protein and possesses a growth-promoting potency comparable to that of the bovine hormone. By the same procedure, 29 mg of the hormone could be obtained from 1 gm of lyophilized monkey pituitaries.[4] Thus, it appears that the growth hormone content is higher in the human pituitary than in the monkey gland. Recently it has been estimated that each human pituitary contains 3.6–6.0 mg somatotropin (25, 26).

Biological Activity. Somatotropins from human and monkey pituitaries isolated by the procedure outlined above have been assayed for their growth-promoting activity according to the stimulation of the width of uncalcified tibial cartilage in hypophysectomized rats; the results indicate that their specific activities are comparable to that of the bovine hormone (23). Figure 3 presents a plot of dose-response curve for the human hormone; the relationship of the tibia width (W, μ) to the function of the total dosage (D, μg) may be represented by the equation, $W = 42.5 \log D + 184.4$. Thus, a total dose of

[3] We are indebted to Drs. R. Luft and H. Olivecrona of Stockholm for human pituitaries which were obtained by surgical removal from cancer patients. In the later part of our investigations, pituitary glands were obtained from autopsy within 3 days after death; in this connection, it is a pleasure to acknowledge, with thanks, the cooperation and support of Drs. H. D. Moon of San Francisco and C. A. Gemzell of Stockholm. The average weight of a single human pituitary after lyophilization is 193 mg.

[4] Owing to the recent active poliomyelitis vaccine program, a large number of monkeys have been available as a source of pituitaries; and through the cooperation of Otto K. Behrens of Eli Lilly Laboratories, monkey pituitaries were generously put at out disposal. The average weight of a single monkey pituitary after lyophilization is 9.4 mg.

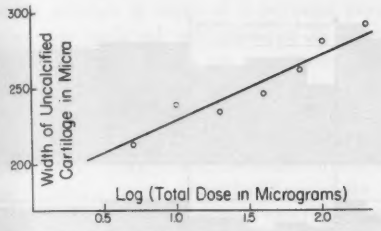


FIG. 3. Effect of human growth hormone on width of proximal epiphyseal cartilage of the tibias of hypophysectomized rats.

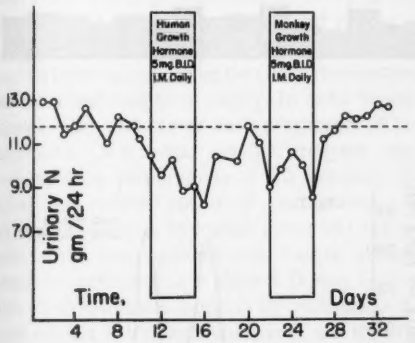


FIG. 4. Effect of human and monkey growth hormone on urinary nitrogen of a human subject with hypopituitarism. No maintenance therapy; nitrogen intake, 13.3 gm/24 hr.

5 μg of the human somatotropin[5] in 4 days would cause an increment of the width of uncalcified cartilage from 160 to 214 μ.

As has already been mentioned, bovine growth hormone is not effective in man. Dr. D. M. Bergenstal of the National Cancer Institute at Bethesda has recently administered our preparations of human and monkey growth hormones to a patient with mild hypopituitarism and to a hypophysectomized male subject. In both cases, a daily dose of 10 mg of either human or monkey somatotropin, injected intramuscularly for 5 days, caused a marked retention of nitrogen and phosphorus indicative of the anabolic nature of the hormone. These preliminary data may be seen in figures 4 and 5. It would be of great importance if it were to be ascertained that primate somatotropins are active in promoting growth in dwarf human subjects.

Molecular Weight. The sedimentation behavior of both monkey and human somatotropins have been investigated in a Spinco Model E ultracentrifuge; in each instance, the hormone sediments as a well-defined and single peak. From these sedimentation data, as well as from the diffusion coefficients, which were determined in a Spinco electrophoresis-diffusion apparatus, and from values for partial specific volume, which were computed from preliminary amino acid data (23), the molecular weights for human and monkey somatotropins were calculated to be 27,100 and 25,400 respectively. These values differ greatly from the molecular weight of the bovine hormone (11). Recently, Ehrenberg and Heijkenskjöld (27) reported similar differences between the molecular weights of human and bovine growth hormones.

Isoelectric Point. The electrophoretic mobility of both human and monkey somatotropins as a function of pH in buffers of 0.1 ionic strength may be seen in figure 6. It is evident that the isoelectric point of the primate hormones is more acidic than that of the bovine hormone. Moreover, the isoelectric point of human hormone is even more acidic than that of monkey somatotropin; this appears to be consistent with the difference in the amino acid compositions of the primate hormones previously reported (23).

Table 3 summarizes some of the physicochemical properties of somatotropins isolated from bovine, monkey and human pituitary glands. The data on the tyrosine and tryptophane content and the dissociation constants (pK_i) of the tyrosine residues in these hormone proteins were taken from the unpublished work of Dr. J. Leonis. It is to be noted that values reported here for the tyrosine content are considerably higher than those reported earlier (23). It may also be seen that the monkey hormone contains four residues of cystine whereas the human contains only two. In this respect, the monkey hormone is more like the beef somatotropin.

C-Terminal Amino Acid Analysis. When hu-

[5] One of the most common biologically active contaminants in bovine growth hormone preparations is thyrotropin (TSH). It has been postulated by some investigators that the inability of actually demonstrating the growth-promoting effects of the bovine hormone in man is perhaps due to the TSH contamination. Dr. R. W. Bates at the National Institute of Arthritis and Metabolic Diseases has assayed two preparations of the human somatotropin by the I^{131}-uptake procedure in chicks and has found that they have a TSH activity of 0.013–0.016 units per mg. If we assume that the pure TSH has an activity of 15 units per mg, the TSH contamination in the human somatotropin is indeed small.

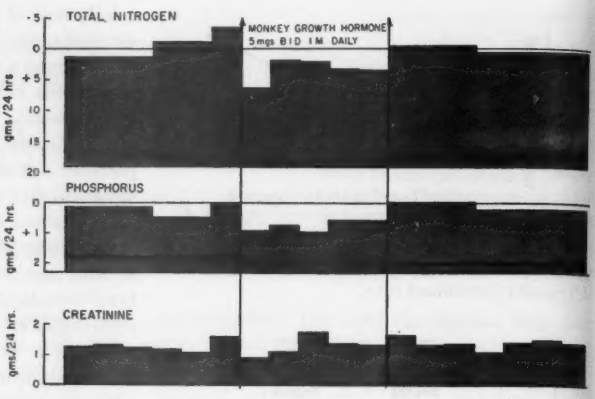
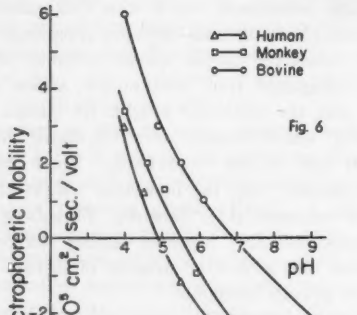


FIG. 5. Effect of monkey growth hormone on urinary nitrogen, phosphorus and creatinine of a hypophysectomized male human subject (cortisone, 50 mg daily and tri-idothyreonine, 12.5 γ daily).



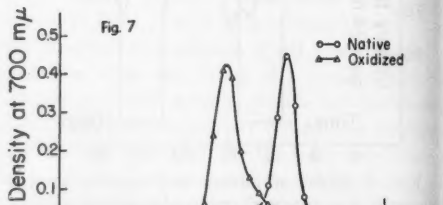


FIG. 6. Electrophoretic mobility of bovine, human and monkey growth hormones as a function of pH at 1° C in buffers of 0.10 ionic strength.

FIG. 7. Zone electrophoresis on starch of performic acid-oxidized and untreated monkey growth hormone in pH 9.95 glycine buffer of 0.1 ionic strength at a potential gradient of 5 γ/cm for 16 hr.

man somatotropin is incubated with carboxypeptidase under the conditions employed for the bovine hormone (16), 1 mole of phenylalanine is liberated per mole of the hormone protein, and smaller amounts of leucine, tyrosine and alanine are progressively released (28). The liberation of the phenylalanine is complete in 2 hr. whereas leucine, tyrosine and alanine are released much more slowly and are found to be present to an extent of less than 0.3 mole per mole of the hormone protein after 6 hr. of digestion. From these data, it may be concluded that human somatotropin has only one polypeptide chain with the C-terminal amino acid sequence . . . Ala(Tyr, Leu)Phe. This conclusion was supported by the results derived from the hydrazinolysis of the hormone protein. It was found that reactions of human somatotropin with hydrazine according to the procedure of Akabori *et al.* gave evidence for phenylalanine as the sole C-terminal residue in the hormone protein (28).

Similar investigations have also provided information for the C-terminal amino acid sequence of the monkey hormone as . . .(Ala, Gly)Phe. In both cases, when the hormone that had lost one residue of phenylalanine in the C-terminus through the action of carboxypeptidase was assayed for biological potency, it was found to have retained its growth-promoting activity (28). It may be recalled that the C-terminal phenylalanine is also not essential for the biological functions of bovine somatotropin (16).

N-Terminal Amino Acid Analysis. Hydrolysis of dinitrophenylated (DNP-) derivatives of either human or monkey somatotropin was found to yield DNP-phenylalanine as the only α-DNP amino acid. Quantitative estimation of the DNP-phenylalanine in the hormone protein indicated

that the number of moles of N-terminal phenylalanine per mole of somatotropin is close to unity (28). These results are in agreement with the conclusion derived from C-terminal amino acid analysis to the effect that the human and monkey somatotropin molecules are composed of a single polypeptide chain. Reaction of the hormone proteins with phenylisothiocyanate afforded additional evidence that phenylalanine is the N-terminal residue in both somatotropins (14).

The fact that dinitrophenylation of both human and monkey hormone protein yielded phenylalanine as the sole N-terminal residue in an amount of 1 mole per mole of somatotropin may be taken as indicating that both preparations possess a high degree of purity. In order to substantiate the validity of such a criterion of homogeneity, N-terminal residue analyses were performed on preparations of the hormone obtained at various stages of purification and possessing different biological potencies; the results of the investigations with human somatotropin are summarized in table 2. It may be seen that phenylalanine is found in all fractions as the predominant N-terminal amino acid, and that the other N-terminal amino acids (i.e., alanine, tyrosine, valine, glutamic and aspartic acid) found in smaller amounts disappear in the final step of purifications. Thus, these data lend further support to the conclusion that phenylalanine is the N-terminal residue of the hormone protein.

Oxidation With Performic Acid. From these analytical data, it may be postulated that both human and monkey somatotropins are composed of a single polypeptide chain with phenylalanine as the terminal residue at both the C- and N-termini. Since the human and monkey hormones have 2 and 4 cystine residues (23) respectively, it might be anticipated that all —S—S— bridges are located intramolecularly along the polypeptide chain. This was found to be the case when performic acid-oxidized somatotropins were submitted to electrophoresis and ultracentrifugation. It was found that all the cystine residues had been converted into cysteic acid, and only one component was revealed by zone electrophoresis on starch (fig. 7). Moreover, the oxidized hormone sedimented in the ultracentrifuge as a single protein with a sedimentation coefficient nearly identical to that of the untreated hormone. There are no indications of a decrease in the molecular size nor of the formation of two or more smaller molecular species after the cleavage of —S—S— bridges by oxidation with performic acid in either somatotropin. Hence, it is reasonable to assume that human and monkey growth hormone have a generalized gross structure as shown in figure 8.

TABLE 3. PHYSICOCHEMICAL CHARACTERISTICS OF GROWTH HORMONE ISOLATED FROM HUMAN, MONKEY AND BEEF PITUITARY GLANDS

Physicochemical Characteristics	Beef	Monkey	Human
Sedimentation constant, ($S_{20,w}$)	3.19S	1.88S	2.47S
Diffusion constant, $D_{20} \times 10^7$	7.23	7.20	8.88
Molecular weight	46,000	25,400	27,100
Isoelectric point, pH	6.85	5.5	4.9
Tyrosine, residues/mole	12	10	8
pK_i	10.4	10.2	9.8
Tryptophan, residues/mole	3	1	1
Cystine, residues/mole	4	4	2
Methionine, residues/mole	7	6	4
Total amino acid residues	396	241	245

Is the Integrity of the Protein Molecule Essential for Hormonal Activity? As has already been mentioned, the removal of C-terminal phenylalanine from either human or monkey somatotropin by the action of carboxypeptidase does not cause loss of growth-promoting activity. It is also possible to show that limited digestion of both somatotropins with chymotrypsin does not lead to inactivation. From the data summarized in table 4*A* and *B*, it is evident that hydrolysis of human somatotropin with chymotrypsin to the extent of 10% did not cause inactivation, but longer digestion did diminish the biological activity. On the other hand, the monkey hormone could be digested to 19% without change of the growth-promoting potency; as expected, furthe digestion with the enzyme was found to result in the loss of the biological activity. As in the case of bovine somatotropin (11), these results appear to indicate that the integrity of the intact protein is not essential for the activity of human and monkey growth hormone.

It is of interest to note that the extent to which hydrolysis of the three somatotropins could be carried out without inactivation decreases in the following order: bovine (25%) > monkey (20%) > human (10%). The rate of chymotryptic digestion of the three hormone proteins as measured in a Jacobsen-Leonis pH stat may be

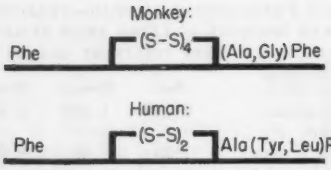


FIG. 8. Proposed structure for monkey and human growth hormones.

TABLE 4. ACTION OF CHYMOTRYPSIN ON HUMAN AND ON MONKEY GROWTH HORMONES

Time of Digestion*	Non-prot. N	Bioassay†	
		No. rats	Tibia width‡
min.	%		μ
A. Human Growth Hormone			
0	0	7	239±4
160	10	8	233±2
420	21	9	203±4
B. Monkey Growth Hormone			
0	0	5	243±2
30	6	5	244±1
70	13	7	229±3
120	19	10	233±4
180	24	8	216±4
300	34	13	186±6

* Enzyme-hormone ratio (wt. basis) = 1:300, pH 9.5 at 25°C. † A total dose of 40 μg in 4 days. ‡ Mean ± standard error.

seen in figure 9. It is evident that human somatotropin is the most resistant to the action of chymotrypsin whereas the bovine hormone is the most susceptible. These differences in the rate of hydrolysis with a proteolytic enzyme may also be taken as an indication of dissimilarity in composition and structure. These observations provide an additional manifestation that the three somatotropin molecules differ completely from one another with respect to chemical and structural characteristics. The hormones from all three species are equally active, however, in promoting growth in rats, and only the bovine hormone is ineffective in man. Does this mean that the rat is capable of degrading the bovine hormone to the active core, whereas humans do not possess this ability? If this is true, one may assume that the three somatotropins possess an identical core which is the structural center responsible for the growth-promoting activity.

The idea that a hormone molecule possesses an active core in which resides one of its major biological activities is not new. Our recent work, as well as that of others, on the structure of ACTH (11) and intermedin (MSH) (29) has indicated that the intrinsic melanocyte-activity in the ACTH peptide molecules may reside in a

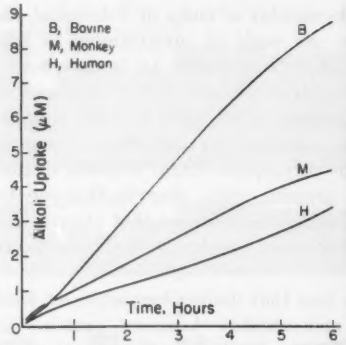


FIG. 9. Alkali uptake accompanying hydrolysis of bovine, monkey and human growth hormones with chymotrypsin at pH 9.5 and 25° C; borate buffer of 0.1 ionic strength; hormone, 10 mg and enzyme, 0.033 mg in 4-ml solution.

sequence of from 7 to 11 amino acids also found in MSH. Moreover, it is now well established that 11 amino acids at the C-terminus of the corticotropins are not necessary for the ACTH activity. All this evidence points to the presence in all ACTH preparations of an identical active core. It is therefore not unreasonable to assume that the bovine growth hormone can be degraded enzymatically or chemically to an active core which it has in common with the monkey and human growth hormone molecules.

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STRUCTURAL BASIS OF RIBONUCLEASE ACTIVITY

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At the annual meeting of the Federated Societies in 1956, Dr. Stanford Moore presented (1) a summary of the status, at that time, of the structural investigations of ribonuclease proceeding at the Rockefeller Institute and at the National Heart Institute. I would like to summarize in this discussion some of the advances of the past year in our knowledge of both the structure of ribonuclease and of the structural basis of its catalytic activity.

Figure 1 summarizes much of what we know at present about the amino acid sequence of ribonuclease. This protein consists of 124 amino acid residues. These residues are linked together in a single chain, as evidenced by the fact that there exists only one N-terminal and one C-terminal residue in the protein and by the fact that the molecular weight, after oxidation of disulfide cross-linkages, is still essentially the same as that of the native molecule. Ribonuclease contains 8 half-cystine residues, all of which are present in disulfide linkage. The formula in figure 1 represents the extended chain produced by oxidation of the 4 disulfide bridges and it is on this oxidized derivative that nearly all of the sequential work has been carried out. A number of investigators concur in the fact that there exist no free sulfhydryl groups, although Ledoux has presented evidence (2), both chromatographic and chemical, that such groups do exist in the protein. We have not, to the present, been able to confirm these findings.

In the time available, we cannot hope to discuss in any detail the methods and specific results obtained in the sequence studies. I would like to point out, however, that the group at the Rockefeller Institute, which includes Drs. Werner Hirs, Stanford Moore and William Stein, has hydrolyzed the extended chain with several different proteolytic enzymes, the sites of cleavage of which are indicated, and has then reconstructed the molecule by a consideration of the overlaps in composition between the different peptide fragments produced (3, 4). In our own studies, we have purposely restricted the number of cleavages by masking the ϵ-amino groups of lysine residues with either dinitrophenyl or carbobenzoxy- groups and have then permitted the enzyme, trypsin, to attack the peptide bonds following the 4 arginine residues which are scattered throughout the chain. Reassembly of the fragments is then possible by consideration of the sequences in the neighborhood of these arginine residues, the nature of which was determined on arginine-containing peptides isolated from hydrolysates prepared either by acid hydrolysis or by general proteolytic degradation (5). The sequence as shown is a summation of both the data of Hirs, Moore and Stein and our own. The continuation of the sequence studies is in progress at the Rockefeller Institute by systematic chemical degradation of the peptide fragments obtained earlier (6), and at Bethesda, by structural studies on fragments produced either

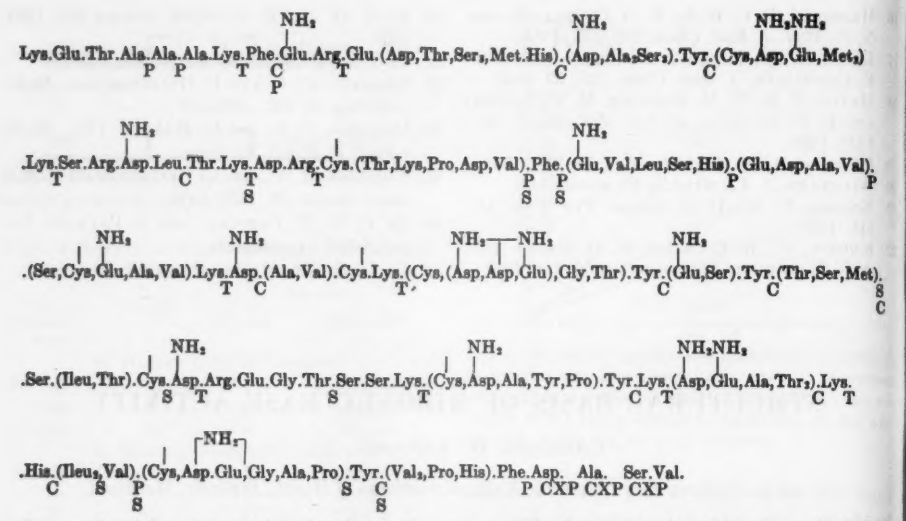


FIG. 1. Partial reconstruction of amino acid sequence of bovine pancreatic ribonuclease (3, 4, 5, 7). Pepsin, *P*; trypsin, *T*; chymotrypsin, *C*; subtilisin, *S*; carboxypeptidase, *CXP*. The symbol, NH_2, indicates that glutamine or aspartic acids exist as their amides.

during studies on disulfide bridges (7) or on peptides produced during enzymatic digestion in the course of studies on the active center of the enzyme. We have, in some instances, been dealing with different areas of sequence, but quite often, with the same peptide portions and much of the available sequence data has thus had double checking.

Of critical importance to our future understanding of the three-dimensional form of ribonuclease in solution is the location and nature of the disulfide bridges. Drs. Spackman, Moore and Stein are presenting, at these meetings, some of their results in this regard (8). We have also been concerned with these bonds and have recently been able to elucidate their structure (7), two of them in complete agreement with the results of Dr. Spackman and his colleagues. In our studies we have employed the enzyme, subtilisin, to degrade the native ribonuclease molecule to a mixture of small peptides from which those containing disulfide bridges could be isolated by filter paper electrophoresis. Figure 2 shows the sort of pattern obtained upon such electrophoresis. Four of the ninhydrin-positive bands shown here gave positive reactions with the cyanide nitroprusside stain of Toennies and Kolb (9). After elution and purification by further electrophoresis, these were shown to contain the amino acids listed in table 1. Each disulfide-positive band yielded, upon oxidation with performic acid, 2 cysteic-acid containing peptides, the compositions of which are shown here. Without taking the time to show how these fragments may be fitted into the available sequence data, the results establish the presence in ribonuclease of 4 disulfide bridges linking, respectively, half-cystine 1 with half-cystine 6, 4 with 5, 3 with 7 and 2 with 8. I should point out that we all look forward to the completion of similar studies by Spackman and his collaborators, since there is always present the haunting possibility that unexpected interchange reactions or other rearrangements may have taken place under any given set of conditions. It is gratifying, however, that bridges 1–6 and 4–5 appear to be demonstrable in both laboratories. For the sake of discussion, we have constructed, as shown in figure 3, a two-dimensional arrangement of the amino acid residues in ribonuclease, drawn in such a way as to indicate more clearly certain interesting features of the molecule. In this figure we have abbreviated glutamine and asparagine as Gn and An, respectively, to save space. Half-cystine residues are marked in red, positive charges in blue, negative charges in yellow, and tyrosine residues in green. Heavy circles surround residues in known sequence. The enzyme has an N-terminal 'tail' of 25 residues and a C-terminal 'tail' of 11 residues.

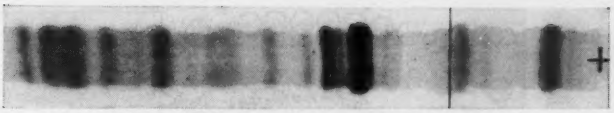


FIG. 2. Pattern obtained upon electrophoresis of a subtilisin digest of ribonuclease.

TABLE 1. CYSTEIC ACID-CONTAINING PEPTIDES PRODUCED BY PERFORMIC ACID OXIDATION OF CYSTINE-CONTAINING PEPTIDES ISOLATED FROM SUBTILISIN DIGESTS OF NATIVE RIBONUCLEASE

Disulfide-Positive Band	Composition	Half-Cystine Residues Implicated	Disulfide Bridge
A	(1) $CySO_3$-Asp-Arg-Glu_2(Ser_2, Thr, Gly)	6	1–6
	(2) ($CySO_3$, Asp, Glu)	1, 3, 5, 8	
C	(1) (Ser, Glu, $CySO_3$)-Lys	3	3–7
	(2) (Gly, Asp, $CySO_3$, Pro, Ala, Lys, Tyr)	7	
D	(1) $CySO_3$-[Ala, Tyr]	8	2–8
	(2) Asp-Arg-$CySO_3$-(Pro, Lys, Asp, Val, Thr)-Phe	2	
E	(1) (Gly, Asp, $CySO_3$, Pro, Ala, Lys, Tyr, Ser)	7	4–5
	(2) ($Ser_{1.1}$, $Glu_{2.0}$, $Asp_{2.7}$, $Lys_{1.8}$, $Tyr_{0.9}$, $CySO_{3\ 3.2}$, $Gly_{0.7}$, $Thr_{0.6}$, $Ala_{1.0}$, $Val_{1.0}$)	3, 4 and 5 together	

both of which are outside any cross-linked area. It is of interest that 3 of the 4 proline residues and 2 of the 4 histidine residues are located within the last 20 residues in the chain. The loop including half-cystines 4 and 5 is drawn here as containing only 5 residues, but I should hasten to point out that this is quite schematic, since the exact sequence, including one of the half-cystine residues, is unknown in this area for a distance of 4 amino acids.

There are, of course, two obvious major aims beyond the elucidation of the complete sequence itself. These are, first, the correlations between structure and physicochemical behavior and second, correlations between both covalent and noncovalent structure and biological activity. I would like, first, to discuss some of the chemical modifications which yield derivatives of interest in connection with the activity question and subsequently some of the provocative physicochemical properties of the native molecule and its derivatives.

Of considerable interest in connection with the active center are the fundamental experiments of Dr. Fred Richards (10), who has shown that the enzyme, subtilisin, under controlled conditions, can remove most, if not all, of the N-terminal, uncrosslinked, portion of the molecule without causing detectable loss in enzyme activity. It has also been observed both by Dr. Richards and myself (unpublished) and by Kalnitsky and Rogers (11), that carboxypeptidase can remove the C-terminal valine residue and probably the preceding serine and alanine residues as well, without activity loss. Indeed, in the case of carboxypeptidase, Rogers and Kalnitsky (12) have shown that with preparations of this proteolytic enzyme, which probably contained contaminating activities of other sorts, a considerable number of residues may be removed from more or less undetermined portions of the molecule without decrease in biological function.

The converse of such studies has been the attempt to determine the minimum change in the molecule necessary to cause inactivation. It has been shown by Weil and Seibles (13) that photooxidation of what appears to be a single histidine residue leads to complete inactivation. In a similar vein, we have found that limited pepsin digestion also causes complete loss of activity (14). During the course of pepsin digestion a tetrapeptide fragment is released, whose structure fits exactly with the C-terminal tetrapeptide sequence of the enzyme. Upon closer examination of this process it was observed that the loss in activity could be correlated quite closely with the rate at which the tetrapeptide appeared (fig. 4). It was then possible to isolate from such pepsin digests an inactive derivative of ribonuclease in good yield which, we may conclude for a number of reasons, differs from the inactive enzyme only in lacking the four amino acids that are C-terminal. First, the inactive derivative contains C-terminal phenylalanine (15). Second, upon oxidation and rupture of disulfide bridges it still has the same molecular weight. Finally, no other N-terminal amino acids can be demonstrated by a variety of end-group methods.

Another covalent modification of interest in connection with the question of the active center

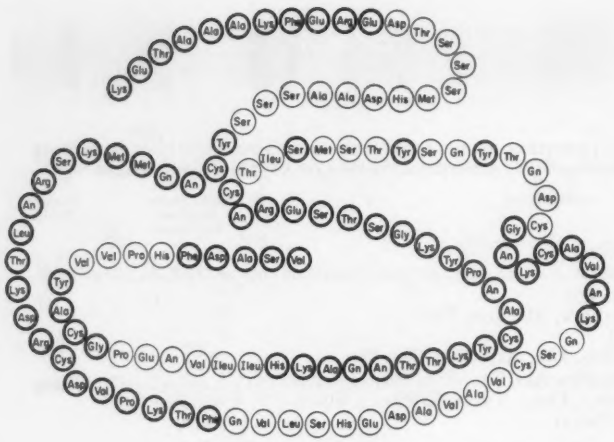


FIG. 3. Schematic diagram of structure of bovine pancreatic ribonuclease; *An*, asparagine; *Gn*, glutamine. *Heavy circles*, known sequence.

of ribonuclease has been the stepwise reduction of the disulfide bridges by thioglycollic acid. Drs. Sela and White and myself (16) have recently found, in analogy to the results of Lindley (17) with insulin, that thioglycollic acid in the absence of urea leads to the rapid reductive cleavage of two of the four SS bridges in the molecule, as evidenced by the fact that 4 —SH groups are quickly produced. Subsequent reduction of the third SS bridge appears to proceed very slowly in the absence of urea and only in the presence of 8 M urea are all the disulfide bridges cleaved. It has been possible to estimate quantitatively the number of disulfide bridges present in the active molecule during the course of such reduction experiments, either by titration with paramercuribenzoate or by alkylation of the —SH groups with iodoacetic acid, the latter procedure leading to S-carboxymethylcysteine groupings. The number of such S-carboxymethylcysteine residues in the molecule could then be estimated by chromatography of the dinitrophenylated hydrolysate of the protein.

Figure 5 shows some of the results obtained during the reductive cleavage of native ribonuclease. In this slide the activity of the product is plotted against the number of sulfhydryl groups present in the average molecule. The points indicated by solid circles are those produced in the presence of 8M urea, whereas, the solid triangles represent samples obtained in the absence of urea. The open symbols represent the activities and sulfhydryl group content of samples of fully reduced or nearly fully reduced material which has been subjected to reoxidation with

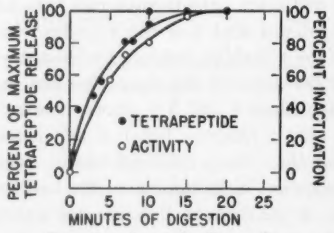
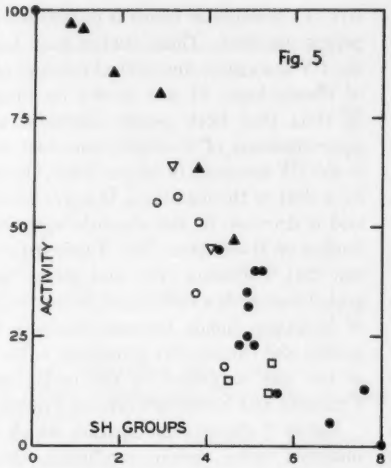
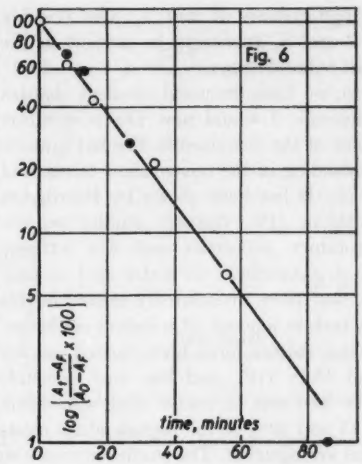


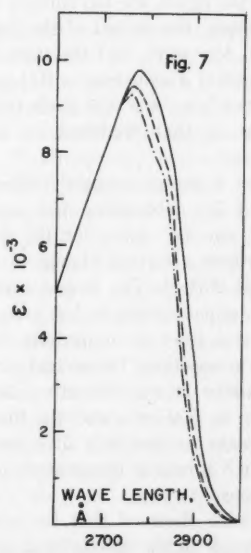
FIG. 4. Inactivation of ribonuclease and appearance of the tetrapeptide, Asp-Ala-Ser-Val, during pepsin digestion at pH 1.8.

molecular oxygen. We have found, in general, that the fully reduced protein containing 8 sulfhydryl groups per mole can be activated to the extent of 20–30% by such treatment. I should state, parenthetically, that carboxymethylation of the sulfhydryl groups present in an active reduction intermediate does not lead to loss of the activity present, nor does exposure to the alkylating agent affect the native enzyme.

That enzymatically active, partially reduced substances are, indeed, produced by reductive cleavage is already suggested by the S-shape of the curve drawn through these points. Further, samples of material, containing on the average 4 or so sulfhydryl groups per mole, give no evidence of contamination by native enzyme upon electrophoretic analysis. It will, of course, be of great interest to determine which of the 4 disulfide bridges in the native molecule can be dispensed with, without complete loss in function. We have, so far, carried out only preliminary experiments in this direction but can now hopefully suggest that of the 4, at least, the bridge







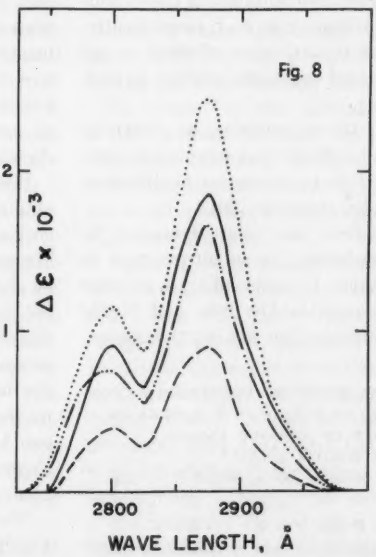


FIG. 5. Changes in ribonuclease activity during reductive cleavage of disulfide bridges; *solid triangles*, reduction with thioglycollate in absence of urea; *solid circles*, in presence of 8 M urea. *Open points* represent results of reoxidation experiments; *open squares*, oxidation with gaseous oxygen of fully reduced (8 SH groups/mole), inactive ribonuclease; *other open points*, oxidation of partially active intermediates. For details see (23).

FIG. 6. First order plot of activity of ribonuclease in buffer (*open circles*) and in 8 M urea solution (*solid circles*).

FIG. 7. Spectra of inactive ribonuclease (*top curve*), of inactive derivative of ribonuclease isolated from limited pepsin digests (*middle curve*), and of an exhaustive pepsin digest of ribonuclease (*bottom curve*).

FIG. 8. Differences in extinction at various wave lengths between native ribonuclease and a complete pepsin digest of ribonuclease (*upper curve*), of the inactive pepsin derivative (*second curve*), of ribonuclease in 8 M urea (*third curve*), and of ribonuclease in 8 M urea in the presence of .003 M phosphate ions (*bottom curve*).

between half-cystines 3 and 7, and possibly between 2 and 8, appear to be present in the active half-reduced enzyme.

Hitherto, we have discussed covalent changes in the molecule. I would now like to mention briefly some of the experiments directed towards an understanding of the noncovalent features of ribonuclease. It has been shown by Harrington and Schellman (18), through studies on the optical rotatory properties and the intrinsic viscosity of ribonuclease in water and in urea solutions, that there undoubtedly existed in this protein a certain amount of α-helical configuration. Similar studies have been carried out by Yang and Doty (19), and one may conclude from these two sets of results that somewhere between 15 and 40% of the peptide chain exists in a helical arrangement. The studies in urea also indicate an extensive disorientation of the secondary structure of ribonuclease and, consequently, it was of interest to determine whether or not the molecule retained enzymatic activity in such a solvent.

It was found, to our surprise, as shown in figure 6, that full activity was retained in spite of what appeared to be enormous modification of hydrogen bonded structure (20).

Some amendments may now, however, be made to this conclusion. As an introduction to these considerations I would like to mention some experiments which Dr. Sela and I (15) have recently performed on the inactive derivative of ribonuclease which is produced by limited pepsin digestion. These studies have to do with the UV absorption and optical rotatory properties of ribonuclease. It was shown by Shugar (21) in 1952 that both pepsin digestion and high concentrations of hydrogen ions lead to a shift in the UV spectrum of ribonuclease, characterized by a shift of the maximum to lower wave lengths and a decrease in the absolute extinction. The studies of Harrington (22), Tanford (23), Scheraga (24), Perlmann (25), and others, have suggested that such a shift might be due to a rupture of hydrogen bonds between tyrosine hydroxyl groups and carboxylate groupings in the protein, of the sort suggested by the earlier studies of Crammer and Neuberger (26) on ovalbumin.

Table 2. Optical rotation and molar extinction, at 2850 Å, of RNase in 8 M urea in presence of various anions (as sodium salts)*

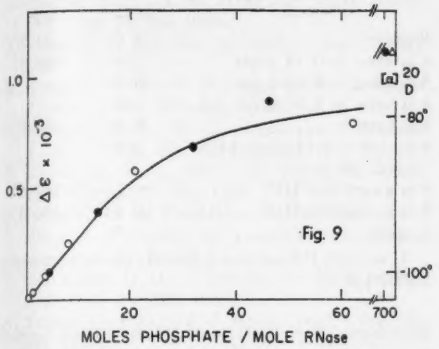
Anion	Molarity	Urea	$\Delta\epsilon \times 10^{-2}$†	$[\alpha]_D^{20}$
Salt-free		8 M	0	−103.7°
Acetate	0.50	8 M	0	
Chloride	0.40	8 M	110	−98.8°
Bromide	0.50	8 M	150	
Sulfate	0.05	8 M		−102.5°
Sulfate	0.25	8 M	840	−88.0°
Arsenate	0.15	8 M	1120	−80.7°
Pyrophosphate	0.15	8 M	1120	
Phosphate	0.001	8 M	112	−103.7°
Phosphate	0.003	8 M	390	−102.9°
Phosphate	0.007	8 M	670	
Phosphate	0.01	8 M	900	−99.9°
Phosphate	0.02	8 M		−96.5°
Phosphate	0.05	8 M		−87.0°
Phosphate	0.15	8 M	1120	−81.0°
Salt-free			1120	−71.7°

* c_{RNase} = 2.45 gm/l for spectrophotometric readings, and 27 gm/l for polarimetric readings.
† Increase relative to spectrum of RNase in 8 M urea in the absence of salts.

Figure 7 shows some spectra which we have obtained under various conditions. On the left part of the figure, the top curve is that of native ribonuclease, the second of the pepsin-produced inactive derivative, and the third of an exhaustive digest of ribonuclease with pepsin. Although not shown here, 8 M urea leads to a shift nearly as great as that produced by limited pepsin digestion.

Figure 8 shows so-called 'difference' spectra obtained by subtracting the various spectral curves from the curve for the active enzyme. We see here a marked change in the extinction at about 2870 Å. The largest shift, represented by the upper curve, is for a complete pepsin digest and is of no immediate interest in the present connection. The second curve represents the inactive pepsin derivative, the third, ribonuclease in 8 M urea and the fourth—and this one I want to discuss a little further—ribonuclease in 8 M urea in the presence of .003 M phosphate ions.

We have observed that the marked changes which occur in the spectral and optical rotatory properties of ribonuclease in strong urea solutions may be fully reversed when small amounts of polyvalent anions are added to such solutions. The spectral properties to which I am referring are those I just discussed, which may involve tyrosine covalent interactions. The rotatory changes are those previously studied by Harrington and Schellman and by Yang and Doty which, according to present theory, may be interpreted in terms of the presence of helical configurations in the molecule under study. Table 2 summarizes, with an unfortunately large number of numbers, some of the data we have obtained in these studies with anions. The



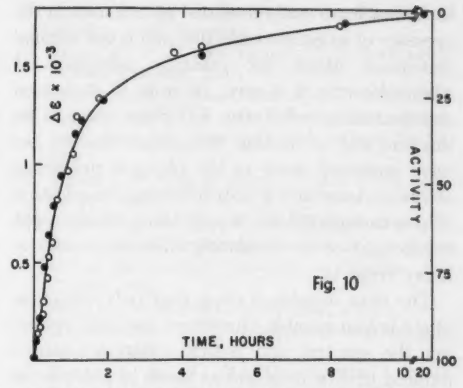


FIG. 9. Effect of phosphate ions on the spectral and optical rotatory properties of ribonuclease in 8 M urea solution.

FIG. 10. Time course of change in extinction and loss of activity during digestion of ribonuclease with pepsin.

specific optical rotation of ribonuclease in water of dilute acetate buffer is $-71.7°$ as measured for the sodium D-line at 20°. In 8 M urea, where helical configurations are presumably absent, this value has increased in the negative direction to $-103.7°$. The molecular extinction of the protein changes 1120 extinction units at 2850 Å in going from aqueous solution to 8 M urea solution. The slide shows further that the addition of arsenate, pyrophosphate, or orthophosphate ions at a level of .15 M completely reverses the spectral change to the level of the native protein in water and nearly completely returns the molecule to its original state in terms of optical rotation. Similar effects may be shown with sulfate ions, although only marked at relatively higher concentrations. Monovalent anions, such as chloride and bromide, show a detectable reversal effect only at extremely high ionic strengths.

Figure 9 shows in greater detail the effect of orthophosphate ions on both the spectral and optical rotatory properties of the protein in 8 M urea solution. Both the spectral and optical rotatory properties have been restored to 50% of the native value when only 20 atoms of phosphorus have been added per mole of protein. This figure of 20, incidentally, is essentially equal to the number of positively charged groups in the molecule, namely, 19. The reversing effect of anions on these physical-chemical properties of ribonuclease is somewhat similar to that previously reported by Simpson and Kauzmann (27) for other molecules, except that the intensity of interaction in this case appears to be some 15-20 times as great and indicates a very special sort of protein-ion interaction.

To return to the derivative produced by limited pepsin digestion, the marked shift in extinction occurring during this direction is of particular interest because of the fact that it is not at all reversed by polyvalent anions, even at high concentrations.

Figure 10 shows the sort of change in extinction that one obtains during pepsin digestion. It is clear from this curve that spectral changes completely parallel changes in enzyme activity. In spite of this evidence for disorientation of the tertiary structure of ribonuclease by pepsin, optical rotatory studies on the purified pepsin derivative show that there has occurred only a negligible change in the optical rotation, indicating minimal changes in the secondary structure of the molecule. The extensive change in extinction, parallelling activity loss, considered in conjunction with the negligible change in optical rotation further supports the conclusion that the chemical consequence of limited pepsin digestion is a very local one and that the molecule as a whole is not seriously modified. The absence of any effect of polyvalent anions on the spectral shift caused by limited pepsin digestion is further evidence for the rather unique involvement of the tetrapeptide fragment removed by pepsin in both the activity and tertiary structural integrity of the enzyme.

Another interesting point raised by the phosphate effect I have just discussed is the following:

Is ribonuclease really unfolded by 8 M urea in the presence of substrate molecules and is our original conclusion about the catalytic adequacy of ribonuclease in 8 M urea, in spite of structural disorientation, valid after all? Since there is no obvious way of testing this point directly, we have measured some of the physical properties of ribonuclease in 8 M urea following the addition of polymetaphosphate, a poly-anion which might be thought of as simulating ribonucleic acid in many respects.

The data in table 3 show that polymetaphosphate is also capable of more or less fully reversing the spectral and optical rotatory changes induced by 8 M urea and at levels of phosphorus atoms per mole very similar to those required with orthophosphte. These results would suggest that ribonuclease is, indeed, refolded, in spite of 8 M urea, in the presence of ribonucleic acid.

As an additional test of this hypothesis, we have investigated the intrinsic viscosity of ribonuclease in 8 M urea under various conditions of environment. The data in table 4 show that the shift in intrinsic viscosity is also extensively reversed by the addition of orthophosphate and that a similar reversal can be brought about by uridylic acid as well. This table contains an additional observation of interest in this connection. Ribonuclease is extensively modified as measured both by viscosity and rotatory measurements by 6 M guanadinium ions. It is of particular interest that the enzyme is completely inactive in such 6 M guanadinium ion solutions in spite of the presence of substrate molecules which, if the situation were analogous to the case of 8 M urea, we would suppose might be completely refolding the molecule. The data in this table show that the viscosity and optical rotatory properties of ribonuclease in 6 M guanadine hydrochloride are, however, not reversed by the addition of orthophosphate.

On the basis of these various data we tentatively conclude, therefore, that there is a correlation between the activity of ribonuclease and the intactness of a portion of its hydrogen-bonded tertiary structure. Thus the data available suggest that the presence of an abnormal spectrum should lead to a diagnosis of inactivity. Guanadine hydrochloride and limited pepsin digestion, both of which lead to spectral changes which are not reversed by phosphate ions, also cause complete inactivation, whereas such agents as 8 M urea and elevated levels of hydrogen ion, causing shifts in spectrum which are reversible, do not lead to loss in activity.

It will be of interest to see whether substrates can cause the refolding of other enzymes, such as papain, which are also active in the presence of hydrogen-bond-rupturing agents.

To summarize, I would like to ask you to look again at the scheme we saw earlier (fig. 3). It appears that the long N-terminal segment of ribonuclease may be removed by subtilisin without impairment of function. Similarly, at least one and probably three of the C-terminal residues may be removed by carboxypeptidase with impunity. Limited pepsin digestion, on the other hand, causing the removal of the C-terminal tetrapeptide sequence and, at the same time, causing a peculiar and irreversible change in tertiary structure, leads to full inactivation without any other obvious modifications of the structure of the enzyme. The involvement of the aspartic residue, at residue 120, in the catalytic center of ribonuclease is strongly inferred. Finally, it appears that perhaps only two of the disulfide bridges need be intact for the expression of enzymatic activity. With these bits of information and with the elucidation of the full sequence of the peptide chain and of the relative importance in catalysis of the individual disulfide bridges, it would begin to appear that the way might soon be open towards an eventual synthetic attack on the structural basis of ribonuclease activity.

TABLE 3. RNASE IN 8 M UREA IN PRESENCE OF POLYMETAPHOSPHATE

P/RNase	$\Delta\epsilon \times 10^{-3}$ at 2850 Å	$[\alpha]_D^{20}$
0	0	−103.7°
8.5	335	
17	447	
34	894	
85	1020	
268		−82.8°

TABLE 4. INTRINSIC VISCOSITY OF RIBONUCLEASE IN VARIOUS SOLVENTS

Solvent	η_{sp}/C	$[\alpha]_D^{20}$
Water	3.6	−71.7°
8 M urea + 0.15 M ph	5.0	−81.0°
8 M urea + 0.02 M ph	5.7	
8 M urea + 0.01 M ph	7.0	
8 M urea	8.5	−103.7°
8 M urea + 0.15 M urydilic acid, pH 6	5.2	
6 M guanidine HCl	9.7	−108.1°
6 M guanidine HCl + 0.15 M ph	10.4	−102.3°

C = 1.4% RNase in 0.1 N KCl; ph = phosphate buffer pH 6.

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STRUCTURE AND ACTIVATION OF TRYPSINOGEN AND CHYMOTRYPSINOGEN

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The present contribution to this symposium will deal with processes involving the formation of a biologically active protein from an inactive precursor, specifically with the formation of the proteolytic enzymes, trypsin and chymotrypsin, from their respective zymogens.[1] These systems are of particular interest since, in consequence of the enzymatic hydrolysis of a limited number of peptide bonds, the biologically inert protein becomes endowed with specific catalytic functions. Viewed in a different light, these transformations may be said to represent the final steps in the biosynthesis of biologically active proteins. The problem of the relation between structure and function of these enzymes may, therefore, be approached by a detailed comparison of the chemical and physical properties of the zymogen before and after activation and by a study of the nature of the changes accompanying the conversion of the zymogen to the active enzyme. The question as to whether the catalytic site is pre-existent in the zymogen molecule or whether it is formed during the transformation is a major aspect of the problem under consideration.

Trypsinogen and chymotrypsinogen have been selected for this discussion since they are the best characterized representatives of the zymogens, as a group, and since, as will be shown, the patterns of their activation and the enzymatic activities of the corresponding enzymes show certain common features. Such differences as have been found here, as well as in other studies of the activation of procarboxypeptidase (1) and pepsinogen (2), have

[1] A more comprehensive review of the subject of zymogen activation, including complete bibliography, may be found in a forthcoming publication (H. Neurath, *Advances in Protein Chemistry*, Vol. 12. In press.)

TABLE 1. PHYSICOCHEMICAL PROPERTIES OF TRYPSINOGEN AND DIP-TRYPSIN

	Trypsinogen	Trypsin
Molecular weight		
S, D	23,800	23,800
Chemical	23,300	
Isoionic point	9.3	10.1
N-terminal	Valine	Isoleucine
C-terminal (Carboxypeptidase, hydrazinolysis)	None	Lysine (?)

served to direct attention to the individual structural characteristics of these proteins. For purposes of organization of the material to be presented, we shall consider separately the activation of trypsinogen and chymotrypsinogen and then attempt to formulate a hypothesis applicable to both systems.

Considering first trypsinogen as a protein, it appears to be homogeneous as judged by sedimentation analysis, electrophoresis and ion exchange chromatography (3, 4). Some of the characteristic physical-chemical properties of trypsinogen are given in table 1, together with those of crystalline trypsin, the latter in the form of the inactive diisopropylphosphoryl (DIP) derivative. Within the limits of the measurements, the molecular weights of trypsinogen and trypsin are the same, indicating that no large fragments are liberated during the conversion. Both proteins have a relatively basic isoionic point, 9.3 for trypsinogen and pH 10.1 for trypsin (5, 6). As judged by end group analysis, both proteins have only one N-terminal group, but significantly, as first shown by Desnuelle and coworkers (7), during the conversion reaction the N-terminal valine of trypsinogen becomes replaced by the N-terminal isoleucine in trypsin. And whereas trypsinogen is devoid of a C-terminal group reactive either toward carboxypeptidase (8) or detectable by the hydrazinolysis method (4), certain preparations of trypsin contain lysine as the C-terminal group (9). The balance of the evidence indicates that both trypsinogen and trypsin consist of a single polypeptide chain.

The amino acid composition of trypsinogen and of DIP-trypsin has been recently determined in collaboration with Miss Cohen in our laboratory and some representative data are presented in table 2. With a few exceptions, the amino acid composition is identical for the two proteins. There are approximately 20 basic groups contributed by 14 lysines, 2 arginines, 3 histidines and one α-amino group. The proteins are particularly rich in aliphatic-hydroxyl groups contributed by 38 serines and approximately 10 threonines. Both proteins contain approximately 12 half-cystine residues, which provide for 6 intramolecular disulfide bridges.

The conversion of trypsinogen to trypsin is an enzyme-catalyzed reaction which, depending on the chosen conditions, can be mediated either by intestinal enterokinase, by penicillium kinase or by trypsin itself (10). In what follows, we shall consider chiefly the activation by trypsin, which at pH 7–8 in the presence of calcium ions follows the kinetics of an autocatalytic reaction and proceeds to completion. Under these conditions, as will have been noted from the preceding two tables, certain changes in the protein occur, including *1*) the replacement of the N-terminal valine by an isoleucine group, *2*) a shift of the isoionic point to a more basic pH, and *3*) the loss of 4 out of 25 aspartic acid residues of the trypsinogen molecule. All these changes are interrelated manifestations of a single event, namely, the splitting of a peptide bond near the N-terminal portion of the peptide chain of trypsinogen, giving rise to the liberation of a hexapeptide which, according to Davie and Neurath (11), has the structure val-$(asp)_4$-lys. The composition of this peptide is in accord with studies by Desnuelle and coworkers (7) which have shown the N-terminal sequence of trypsinogen to include, besides valine, aspartic acid and lysine. The question may now be raised whether the splitting of this isoleucyl-valine bond, with the consequent release of the peptide, is functionally related to the appearance of enzymatic activity, and whether these chemical changes are necessary and sufficient attributes of the activation process. Additional

TABLE 2. AMINO ACID COMPOSITION OF TRYPSINOGEN AND DIP-TRYPSIN*

Amino Acid (Residues/mole)	Trypsinogen	DIP-Trypsin
Arginine	2.0	2.0
Histidine	3.1	3.0
Lysine	14.5	14.0
Aspartic	25.4	21.5
Glutamic	11.0	11.1
Phenylalanine	3.1	3.0
Tyrosine	4.0	4.1
Cystine	6.5	6.4
Proline	8.0	8.0
Serine	39–40	39–40
Threonine	10.9	11.4

* Cohen and Neurath, unpublished.

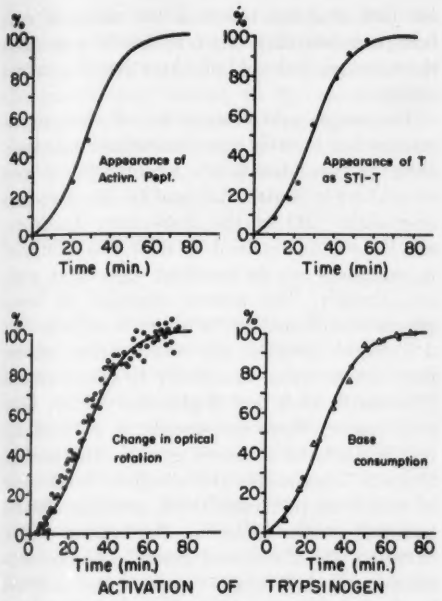


FIG. 1. Correlation of changes occurring upon activation of trypsinogen (4).

experimental evidence relevant to this question is presented in figure 1. In each of the four diagrams shown in this figure, the percentage change in property during activation is plotted against time of activation. The solid line represents the appearance of enzymatic activity, its sigmoidal shape being characteristic for an autocatalytic reaction. In the graph on the top left, the appearance of the N-terminal hexapeptide of Davie and Neurath is seen to follow closely the appearance of enzymatic activity. In the diagram on the top right, the rate of appearance of trypsin, as determined by moving boundary electrophoresis, is compared to the appearance of enzymatic activity and the two parameters can be seen to be fully concordant. In these measurements by Dr. J. F. Pechère (4), activation was carried out in the presence of indole, in order to inhibit spurious chymotryptic activity in the trypsinogen preparations, and soybean trypsin inhibitor was added at various time intervals in order to convert all free trypsin into the soybean trypsin inhibitor compound. It will be noted that trypsin and tryptic activity appear simultaneously throughout the reaction.

The diagram on the bottom right side refers to the determination of the number of peptide bonds split during the activation process, as determined by titrations at constant pH in the pH-stat of Jacobsen and Léonis (12). Since the pK of the newly formed amino group is unknown at the present time, it is not possible to calculate unequivocally the number of peptide bonds opened. However, assuming a pK of 7.6, the titration curves would correspond to the opening of one peptide bond. When the percentage change in hydrogen ions appearing is plotted against the same time axis as the percentage of tryptic activity appearing, the resulting curves are superimposable, suggesting strongly that no peptide bond had been broken other than the bond which gives rise to the liberation of the hexapeptide. The data thus lend strong support to the view that one and only one peptide bond is hydrolyzed during this process. This conclusion is valid within the restriction that the hydrolysis of a peptide bond involving an amino group with a pK higher than 9 or 10 could not be detected by constant pH titrations at pH 8. Thus, opening of a hypothetical peptide bond involving an ϵ-amino group of lysine would escape detection by these measurements.

Neurath, Rupley and Dreyer (13) have recently shown that the activation of trypsinogen, as well as of chymotrypsinogen, is followed by significant changes in the specific optical rotation of the protein, the levorotation decreasing markedly as enzymatic activity appears. The bottom left-hand graph of fig. 1 shows representative data to compare the increase in specific rotation (decreasing levorotation) with the appearance of enzymatic activity. The two parameters appear to follow the same progress curve, the specific levorotation decreasing from $-45°$ for trypsinogen to $-30°$ for trypsin.

At this stage we may, then, summarize the evidence thus far presented by stating that during the activation of trypsinogen, the peptide bond between lysine and isoleucine is opened, giving rise to the hexapeptide valyl-aspartyl$_4$-lysine, and that, as far as is known at present, this is the only bond opened during the activation process. This single hydrolytic event is accompanied by an appreciable decrease in the levorotation of the protein which, in the light of current knowledge on the relation between the structure and the optical rotation of long-chain molecules (14), may be ascribed to the conversion of the zymogen into a more

nearly helical structure. A more detailed interpretation of this phenomenon, in terms of the mechanism of activation, will be deferred until corresponding data for the activation of chymotrypsinogen have been presented. In passing, it should be noted that trypsinogen contains 16 peptide bonds which are potentially susceptible to tryptic hydrolysis, namely, those contributed by 2 arginine and 14 lysine residues. The significant phenomenon that only one of these is actually hydrolyzed during activation will be considered later on. The above scheme of activation is only followed in the presence of calcium ions, whereas in their absence trypsinogen is converted into an 'inert' protein (10). The details of this process are not known, but it seems entirely possible that under these conditions other peptide bonds contributed by lysyl or arginyl groups are opened by trypsin, thus interfering with activation or causing destruction of the active configuration.

Turning now to chymotrypsinogen and its activation process, we may first consider the known properties of chymotrypsinogen as a protein. Several-times recrystallized chymotrypsinogen can be obtained in a homogeneous state, as judged by solubility (10), ion-exchange chromatography (15) and moving boundary electrophoresis (16, 17). Time does not permit a discussion of the cause and nature of inhomogeneities which have been found in batches prepared under otherwise identical conditions. Some of the characteristic physico-chemical properties of chymotrypsinogen are shown in table 3. Five different methods of analysis converge toward 25,000 ± 500 as the most likely molecular weight of this protein (18, 19). Chymotrypsinogen is a relatively basic protein, with an isoionic point of pH 9.4. The protein contains a single N-terminal group, contributed by a half-cystine residue (20), and according to the best analyses, based on the action of carboxypeptidase (21) or on hydrazinolysis (22), the zymogen is devoid of a reactive C-terminal group.

TABLE 3. STRUCTURAL CHARACTERISTICS OF CHYMOTRYPSINOGEN

Molecular weight	
Chemical analysis	25,081 ± 336
Sedimentation, diffusion,	
pH 3.0	24,400
pH 7.5	24,000
Light-scattering	26,100 ± 1,000
X-ray	25,000 ± 800
Osmotic pressure	24,000 ± 500
Isoionic point	9.4
N-terminal	CySS/2
C-terminal (Carboxypeptidase, hydrazinolysis)	None

The amino acid composition of chymotrypsinogen has recently been determined independently in two laboratories, by Dr. Wilcox and co-workers in Seattle (23) and by Dr. Hirs and co-workers (24) at the Rockefeller Institute, and the results obtained by these two groups of investigators are in excellent agreement with one another. The protein contains 20 basic groups contributed by 13 lysine, 4 arginine and 2 histidine residues, and one α-amino group; these are balanced in part by 10 acidic groups (7 aspartic acids and 3 glutamic acids). Like trypsinogen, chymotrypsinogen is particularly rich in aliphatic hydroxyl groups. The protein contains 5 intramolecular disulfide bonds, one of which, as just mentioned, occupies the N-terminal position. Pending clarification of the nature of the C-terminal group in chymotrypsinogen, we shall simply represent the molecule in the form of a closed chain cross-linked by 4 disulfide bridges; and while this representation may prove to be false, it is of no particular consequence for the present discussion, since the terminal groups do not seem to be involved in the sequence of events attending the activation process.

The activation of chymotrypsinogen is mediated by trypsin (10), and can give rise to several enzymatically active components, the pathway and the final product depending on the concentration of trypsin or, more properly, on the rate of activation (25). Operationally, two conditions of activation may be distinguished, usually referred to as rapid and slow, and within the limitations of time available for this discussion, we shall restrict ourselves primarily to the rapid activation, which causes maximum proteolytic activity to be attained after 1–2 hr. of incubation of chymotrypsinogen with trypsin at 0° (25). The nature of the chemical events accompanying the activation of chymotrypsinogen has been studied independently in two laboratories, by Desnuelle and co-workers at Marseilles (7, 26, 28) and by Gladner, Bettelheim and Dreyer in our laboratory in Seattle (6, 17, 21, 27). The results of these two independent investigations have turned out to be in excellent agreement with one another.

The rapid activation of chymotrypsinogen

is a stepwise reaction, as shown below, the formation of the first intermediate, π-chymotrypsin, being mediated by trypsin, whereas the second step, leading to the formation of δ-chymotrypsin, is catalyzed by chymotrypsin itself and can be inhibited by the addition of a specific chymotrypsin inhibitor such as indole or β-phenylpropionate.

$$\text{Chymotrypsinogen} \xrightarrow{\text{Trypsin}} \pi\text{-Chymotrypsin} \xrightarrow{\text{Chymotrypsin}} \delta\text{-Chymotrypsin}$$

Since the three components differ from one another in electrophoretic mobility (17, 27, 28), the rates of appearance and disappearance of each of these components can be determined by moving boundary electrophoresis, which also permits the isolation of the two forms of chymotrypsin in a high state of purity. The progress curves describing the appearance of these components, together with measurements of the enzymatic activity, are shown in figure 2. It will be observed that in the absence of the chymotrypsin inhibitor, π-chymotrypsin behaves as an unstable intermediate, and that the specific activity of each of the two forms is practically the same. End group analyses of π- and α-chymotrypsin, using Sanger's FDNB reagent for amino terminal groups and carboxypeptidase for carboxyl terminal groups, give an indication of the nature of the bonds which had been hydrolyzed during the activation process. Without going into any experimental details, we should like to present in table 4 the results which have been obtained in this investigation. Each of the three components involved in rapid activation contains one N-terminal residue of cystine (27). The component corresponding to π-chymotrypsin contains, in addition, one N-terminal isoleucine, but no additional N-terminal group seems to be formed during the rapid activation process. The rate of appearance of N-terminal isoleucine follows closely the rate of appearance of enzymatic activity (28), and the rate of disappearance of chymotrypsinogen, suggesting that the splitting of the peptide bond involving the amino group of isoleucine is the rate-determining event in the activation of chymotrypsinogen. When rapid activation was allowed to terminate at a stage corresponding to the formation of π-chymotrypsin, no C-terminal group reactive toward Anson's carboxypeptidase could be found. Attempts to identify a C-terminal group by the method of hydrazinolysis have likewise given essentially negative results (29). However, Folk and Gladner (9) have recently reported that 'basic carboxypeptidase' liberates arginine from preparations of DIP-π-chymotrypsin. The replacement of arginine by leucine in the conversion of π- to δ-chymotrypsin, together with a relatively large shift in electrophoretic mobility, suggested that a basic amino acid or peptide was split off during this process from the C-terminal portion of π-chymotrypsin. The isolation and characterization of such a peptide has been independently reported by Dreyer and Neurath (17), and by Desnuelle and coworkers (28), and the peptide has been identified as serylarginine. Since no other peptide is liberated in significant quantities during this reaction sequence, and since the rate of appearance of this peptide follows closely the progress curve describing the rate of appearance of δ-chymotrypsin (fig. 2), it may be concluded that two and only two peptide bonds are hydrolyzed during the rapid activation of chymotrypsinogen, as indicated in figure 3. In this figure, chymotrypsinogen is represented as a cyclic structure held together by 4 intramolecular disulfide bonds, the fifth cystine containing the free α-amino group. All hydrolytic changes occurring during the rapid activation involve the sequence represented

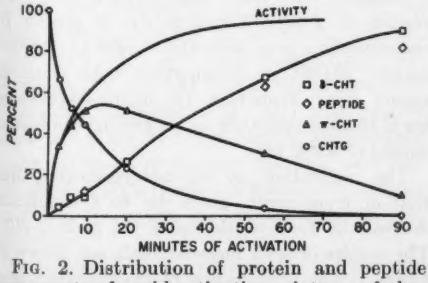


FIG. 2. Distribution of protein and peptide components of rapid activation mixtures of chymotrypsinogen in relation to time and extent of activation (17).

TABLE 4. COMPARISON OF TERMINAL GROUPS OF CHYMOTRYPSINOGEN AND CHYMOTRYPSINS

Protein	N-Terminal	C-Terminal
Chymotrypsinogen	CySS/2	
π-Chymotrypsin	CySS/2	
	Ileu	(Arg ?)
δ-Chymotrypsin	CySS/2	
	Ileu	Leu
α-Chymotrypsin	CySS/2	
	Ileu	Leu
	Ala	Tyr

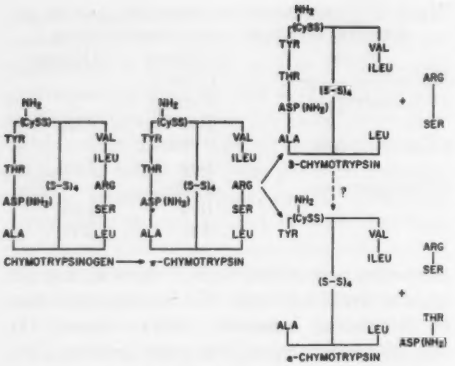


FIG. 3. Proposed scheme for conversion of chymotrypsinogen to δ-chymotrypsin (rapid activation).

arbitrarily on the right of the square, containing adjacently valine, isoleucine, arginine, serine and leucine. The peptide sequence represented on the left side of the disulfide bond is of no consequence for the rapid activation process. It will be seen that, in accordance with its specificity requirements, trypsin hydrolyzes in the first step a peptide bond wherein arginine contributes the carboxyl group, giving rise to isoleucine and arginine as N- and C-terminal groups, respectively. In the second step, catalyzed by chymotrypsin itself, the bond between serine and leucine is broken, giving rise to leucine as the C-terminal group and the liberation of serylarginine.

Mention should be made in this connection of the end product of the slow activation of chymotrypsinogen, slow activation being operationally defined as conditions wherein maximal proteolytic activity appears after 24–48 hr. of incubation at 0° (10). The end product of this reaction is α-chymotrypsin, the protein first described and crystallized by Kunitz and Northrop, and represented on the bottom right-hand side of this slide. Two of the end groups are identical with those found in δ-chymotrypsin, namely, isoleucine and leucine (7, 21, 30). However, in addition, two new terminal groups can be found in this protein, namely, alanine as N-terminal and tyrosine as C-terminal, and as recently shown by Desnuelle and his co-workers, another region in the molecule, represented here to be on the left side of the disulfide bridges, is affected by slow activation (31). The hydrolysis of these two bonds is due to chymotrypsin, the first one to be opened being that between tyrosine and threonine, the second bond, between asparagine and alanine, giving rise to the liberation of the dipeptide threonylasparagine. It has been recently shown (32) that the sequence of events leading from chymotrypsinogen to α-chymotrypsin can be reversed, by first treating chymotrypsinogen with relatively high concentrations of chymotrypsin. The resulting derivatives, referred to as neo-chymotrypsinogens, are all enzymatically inactive but can be activated by trypsin. The interrelations between the group of chymotrypsinogens and the known forms of chymotrypsin are shown in figure 4. Opening of a bond to the left of the cross-linkages so labilizes the chymotrypsinogen that the corresponding product of activation has a specific activity only two-thirds that of π- or δ-chymotrypsin.

As will be noted from this and the preceding figure, α-chymotrypsin consists essentially of two open polypeptide chains held together by 4 disulfide cross-linkages, the fifth disulfide linkage, containing the α-amino group, being somewhere along one of these chains. Some information on the relative length of these peptide chains has been recently obtained by Meedom in Linderstrøm-Lang's laboratory (33), who found that upon oxidation of α-chymotrypsin by performic acid, three fragments are obtained, as shown in table 5. The shortest of these peptides, A, contains the N-terminal cystine and C-terminal leucine. This is presumably the same leucine which in chymotrypsinogen is connected through serylarginine to the isoleucylvaline sequence shown to occupy the N-terminal position in peptide B. The C-terminal group of peptide B is tyrosine which, presumably through the dipeptide, threonylasparagine, is connected in chymotrypsinogen to the alanine which occupies the N-terminal position in peptide C. The C-terminal group of peptide C is unknown. Peptide A contains one cysteic acid; peptide B, 7; and peptide C, 2 (34). The large size of peptide B, corresponding to a molecular weight of approximately 20,000, is incompatible with a recent report that reduction of chymotrypsinogen leads to small subunits of an average molecular weight of 5,000 (35).

The activation of chymotrypsinogen, like that of trypsinogen, gives rise to a significant decrease in the levorotation of the protein (13). The results of such measurements are shown in figure 5. The levorotation of chymotrypsinogen is considerably higher than that of trypsinogen, namely, −80° as compared to −45°, suggesting

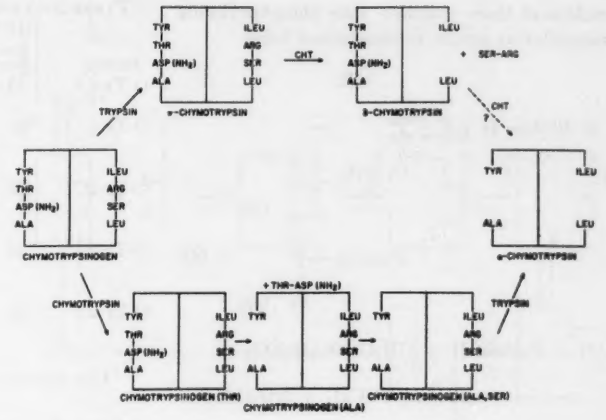


FIG. 4. Interrelations among chymotrypsinogens and chymotrypsins (32).

TABLE 5. FRAGMENTS OF OXIDIZED α-CHYMOTRYPSIN (33)

Peptide A: *Cyst*-(Gly, Glu, Ala, Val_2, Ileu, Pro, Leu)-Ser-Gly-*Leu*. . . Ser-Arg
Peptide B: *Ileu*-Val-(Arg_3, His_2, $(X)_{167}$)-Leu-*Tyr*. . . Thr-Asp(NH_2)
Peptide C: *Ala*-$(X)_{48}$

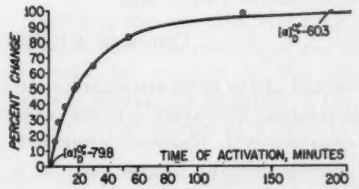


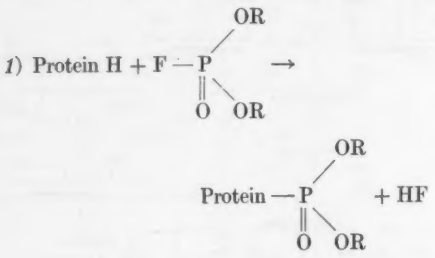
FIG. 5. Changes in optical rotation during rapid activation of chymotrypsinogen (13). *Continuous curve*, optical rotation; *circles*, activity determinations (esterase).

that a relatively small proportion of the molecule is in a helical configuration. The levorotation decreases to a value of $-60°$ as chymotrypsinogen is transformed into π-chymotrypsin. The subsequent conversion of π-chymotrypsin to the δ-form produces no further changes in the rotation, indicating that the splitting of the isoleucylarginine bond is the event which gives rise to the transformation of the molecule into a more nearly helical configuration. Again, as in the case of trypsinogen, chymotrypsinogen contains many bonds which are potentially susceptible to hydrolysis by trypsin; however, only one of these, namely, the isoleucylarginine bond, is hydrolyzed under the action of trypsin, and only 3 additional bonds, previously considered, are susceptible to hydrolysis by the action of chymotrypsin. In accordance with the views expressed by Linderstrøm-Lang and Schellman (36), we shall ascribe this phenomenon of limited proteolysis to the fact that peptide bonds which are hydrogen-bonded are resistant to the action of proteolytic enzymes, and that only those peptide bonds which are not hydrogen-bonded in the native protein are susceptible to proteolysis.

At this stage we may formulate the common features in the activation of trypsinogen and chymotrypsinogen as follows: A single peptide bond, between a basic amino acid and an isoleucyl-valyl-glycyl sequence in a specific region in the zymogen molecule is hydrolyzed by the activating enzyme, namely, trypsin. This leads to a decrease in the specific levorotation of the protein and to the appearance of enzymatic activity. As just stated, only one bond needs to be hydrolyzed in order to produce activation, even though both zymogens contain many more peptide bonds which, on the basis of the substrate specificity of trypsin, would be potentially susceptible to hydrolysis.

A further characteristic feature common to both trypsinogen and chymotrypsinogen is the well known fact that the corresponding active enzymes have specific esterase activity, and that the active site reacts stoichiometrically with certain phosphorylating and acylating agents, such as diisopropylphosphofluoridate or p-nitrophenylacetate. The resulting phosphoryl or acyl enzymes are enzymatically inactive but can be reactivated by nucleophilic reagents (37) in the case of phosphoryl, or in water above pH 6.5 in the case of acyl enzymes (38). The

reaction of these enzymes with phosphorylating and acylating agents is summarized below.



2) *a*, Protein H + $CH_3COOC_6H_4NO_2$ →

Protein $OCCH_3$ + $NO_2C_6H_4OH$

b, Protein $OCCH_3$ + H_2O →

Protein H + CH_3COOH

We should like to focus attention for a minute on this catalytic site involved in the reaction of these enzymes with phosphorylating and acylating agents, since we propose that it is this site which is formed during the activation of the zymogens. Degradation studies on the inactive DIP-derivatives of chymotrypsin, trypsin and liver cholinesterase have shown that in each case the hydroxyl group of serine is the ultimate site of phosphorylation (39, 40, 41). The composition of DIP-peptides obtained by enzymatic hydrolysis of the labeled proteins is rather similar in the three cases, as indicated in table 6. It has been suggested, however, that serine is not the initial focus of interaction between the phosphorylating agent and the enzyme, and various lines of evidence have been adduced in support of the view that the imidazole side chain of histidine is involved in the catalytic function of the enzymes and in their interaction with organic phosphates (42, 43). Time does not permit a critical examination of the arguments

TABLE 6. DIP-PEPTIDES FROM ESTERASES (40)

	α-Chymotrypsin	Trypsin	Liver Aliesterase
DIP	1	1	1
Ser	1	1	1
Gly	2–3	2–3	2
Ala	—	—	1
Asp	1	1	—
Glu	—	—	1
Pro	1	1	1
Leu or Ileu	1	—	1
Val	—	1	—

TABLE 7. PEPTIDE FRAGMENTS OF OXIDIZED DIP^{32}-TRYPSIN (45)

Peptide	Residues	Composition
O-Tr-1	15	Cys_2 Asp_2 Glu Ser_2 Gly_4 Val Lys Pro
O-Tr-3	37	Cys_2 Asp_3 Glu_3(Ser,Gly)$_{14}$ Met Thr Ala_3 Phe Val Tyr Ileu Leu Lys_2 Pro_2
O-Tr-2C	45	Cys_5 Asp_4 Glu_3 Ser_{10} Gly_3 Met Thr Ala_2 Phe Val Tyr Ileu Leu Lys_3 Pro_3
O-Tr-2A	46	Cys_4 Asp_4 Glu_3 Ser_{10} Gly_3 Met Thr_2 Ala_3 Phe Val Tyr Ileu Leu Lys_2 Pro_2 Arg
O-Tr-2B	55	Cys_5 Asp_5 Glu_2(Ser,Gly)$_{19}$ Met Thr_2 Ala_3 Phe Val(Tyr,Ileu, Leu)$_6$ Lys_3 Pro_5 Arg

* Cys denotes cysteic acid.

in favor of such a hypothesis, but, as was shown in a paper presented this morning (44, 45), it is more likely that histidine participates in the phosphorylation and acylation of serine as an intramolecular catalyst rather than being itself a receptor. Nevertheless, histidine and serine must maintain adjacent positions in the active protein molecule, but all attempts to identify histidine in DIP-peptides, obtained by degradation of DIP-trypsin, have failed. This is documented in table 7, which shows a series of large peptides obtained by tryptic hydrolysis of oxidized DIP-Trypsin, labeled with radioactive phosphorus in the DIP moiety (46). Each of the peptides listed in this figure is probably derived from the largest peptide shown on the bottom line. None of these contains histidine, and it is significant that five of the twelve half-cystines are found in a peptide (O—Tr—2B) comprising only one-fifth of the mass of the molecule. Alternatively, however, it may be suggested that serine and histidine are being brought into juxtaposition by the close apposition of two separate parts of the polypeptide chain, and this seems indeed to be supported by the findings that the phosphorylation (47) or acylation (48) of chymotrypsin is abolished by prior denaturation of the protein in 8 M urea. Similar observations have been made for the acylation of trypsin, leading to the conclusion that the active enzymes owe their catalytic function to a favorable and specific orientation between a serine and histidine side chain.

Recently, Cunningham (49) has suggested a mechanism which could account for the joint participation of histidine and serine in both the acylation and deacylation reactions of proteolytic

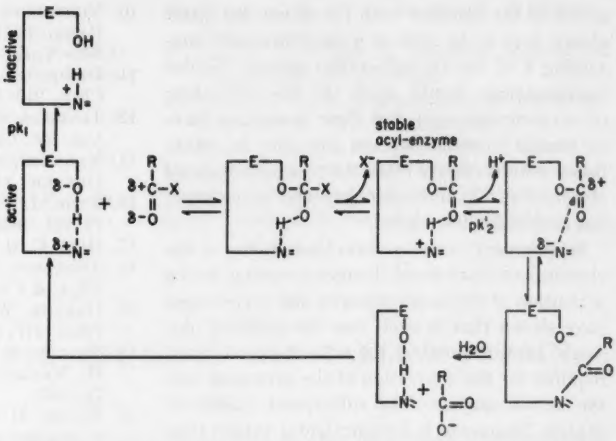


FIG. 6. Proposed mechanism of enzymatic hydrolysis by trypsin and chymotrypsin (49).

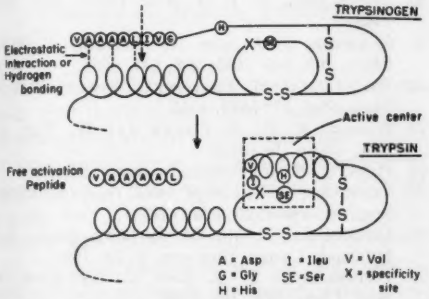


FIG. 7. Schematic representation of structural changes involved in tryptic activation of trypsinogen.

enzymes. This scheme, somewhat modified, is presented in figure 6, and indicates the active configuration to be dependent on the maintenance of a hydrogen bond between these two residues, as shown in this figure. This bond involves the unionized form of the imidazole nitrogen and thus accounts for the pH-dependence of the acylation (44, 45, 50), or phosphorylation (51) reactions. The complex is stabilized at low pH by the formation of a hydrogen bond between the seryl oxygen and the carbonyl oxygen of the substrate. At higher pH this hydrogen bond is broken by loss of a proton. The pH-dependence of the deacylation reaction is thus accounted for by the formulations shown on the right-hand side of this figure. When the stable acetyl-δ-chymotrypsin is denatured by urea at low pH, the acetyl group, which is reactive toward hydroxylamine in the native state, loses this reactivity but is retained on the protein molecule and regains its reactivity toward hydroxylamine upon removal of the urea (48). This again would be consistent with the removal of the histidyl residue from the vicinity of the acyl-seryl residue, thus altering its reactivity in the direction of that characteristic of a simple oxygen ester.

Returning now to the mechanism of activation, it is proposed that only a portion of the molecule is affected by the activation process itself, and that the breaking of a strategically-located peptide bond converts a closed region of the molecule, often referred to as a loop, into an open chain structure. If it is now assumed that by virtue of side-chain interaction the newly formed chains assume a more nearly helical configuration, the decrease in levorotation might be fully accounted for without having to invoke a reorientation of the entire structure of the protein molecule. This opening of the loop at the same time allows a serine and a histidine side chain to come into sufficient proximity in order to interact by hydrogen bonding in a manner such as postulated by Cunningham. This picture might best be represented by a schematic model, which is shown in figure 7 for the activation of trypsinogen. It is proposed that the N-terminal region of the molecule, containing four adjacent aspartic acid side chains, does not fit into a helical configuration and that interaction between the aspartyl carboxyl groups, electrostatically with positively-charged groups or by hydrogen bonding with tyrosyl groups elsewhere along the chain provides a loop which is destroyed when the lysyl-isoleucine bond is broken by trypsin. The N-terminal region of the trypsin molecule then reorients itself so as to establish the inter-

action of the histidine with the serine, the latter shown here to be part of a rigid structure containing 4 of the 12 half-cystine groups. Similar considerations would apply to the activation of chymotrypsinogen, but these postulates have to remain hypothetical and can only be established with certainty after the three-dimensional structure of chymotrypsinogen and trypsinogen has been fully determined.

In summary, we may state that studies of the chemical and structural changes occurring during activation of chymotrypsinogen and trypsinogen have shown that in each case the splitting of a single peptide bond is the sole chemical event required for the conversion of the zymogens into the active enzyme. The subsequent release of peptide fragments is a coincidental rather than an obligatory aspect of the activation reaction. As a result of the splitting of a closed region of the molecule, the newly-opened peptide chains reorient themselves so as to lead to a more nearly helical configuration in the molecule, as evidenced by a decrease in the specific levorotation of the protein. This reorientation, in turn, permits a histidine and serine side chain to interact with one another such as to establish the catalytic site on the enzyme molecule. Specific acylating or phosphorylating agents react with this particular serine side chain provided that the histidine is part of the active configuration. While this configuration would be common to both trypsin and chymotrypsin and probably to other esterases, it is clear that the amino acid side chains in specific orientations determine the characteristic side chain specificity of the two enzymes.

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ACTIVE SITE AND STRUCTURE OF CRYSTALLINE PAPAIN[1]

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Our interest in papain stems from the possibility that this is a particularly suitable enzyme for attempting to correlate specificity, mechanism of action and structure. As with most proteolytic enzymes, it is possible to prepare simple substrates for investigations of specificity and kinetics. The unusual stability of papain over a wide range of temperature, pH and alcohol concentration renders it particularly suitable for such investigations. As will be discussed later, the presence of a sulfhydryl group as a part of the active site of the enzyme leads to the possibility of identifying the specific polypeptide structures which determine the specificity and mode of action of this enzyme. Structural studies are possible because homogeneous, crystalline papain can be prepared in reasonable quantities.

Papain is a slightly basic molecule with a molecular weight of about 20,500 as estimated by a variety of methods. The crystalline protein, or better, its crystalline mercury derivative, is essentially homogeneous as determined by physical, chemical, immunochemical and chromatographic methods (1). In this paper, some of the work which has been done on the properties of the active sulfhydryl group of papain will be discussed together with other information which has been obtained regarding the nature of the active site. Some aspects of investigations of the overall structure of the protein, as yet incomplete, will also be presented.

SULFHYDRYL GROUP

The presence of an essential sulfhydryl group in papain presents both advantages and disadvantages. The enzyme is readily inactivated both by traces of heavy metal ions, which combine with the essential SH group, and by autoxidation under aerobic conditions. The enzyme is fully active only under conditions in which contaminating metal ions are bound by the addition of a chelating agent, e.g., Versene (ethylenediaminetetraacetate) or citrate, and a reducing agent, such as cysteine or BAL (British-anti-Lewisite, dimercaptopropanol) is added to maintain the enzyme in the active state (1).

Papain is inhibited not only by heavy metal ions but also by all of the usual reagents which react with SH groups, e.g., *p*-chloromercuribenzoate, iodoacetamide, oxidizing agents, etc. The high affinity of papain for heavy metal ions has been useful in that it has permitted the isolation of a crystalline mercuripapain which is inactive (2). This derivative is more homogeneous than crystalline papain and has the advantage that autodigestion is inhibited. Crystalline mercuripapain contains 1 mole of mercury to 2 of papain and hence has the composition of a dimer although its physical behavior is more complex (3).

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Under chromatographic conditions which permit fully reduced papain to bind the maximal amount of mercuric ion, it has been found that 1 atom of mercury is bound to 1 mole of papain (4). This derivative is inactive but can be reactivated by addition of BAL.

Studies have been made by the spectrophotometric method of Boyer (5) of the affinity of papain for *p*-chloromercuribenzoate (PCMB) under conditions where the enzyme is fully reduced (6). Figure 1 shows the results of such studies with different preparations of crystalline papain. It is evident that in each case less than one SH group per mole of papain can react with PCMB. Moreover, the proteolytic activity, as estimated by the proteolytic coefficient (C_1) for the hydrolysis of benzoyl-L-argininamide, varies with the amount of reactive SH. These results do not imply that papain preparations contain contaminating proteins. Indeed, such preparations do not show significant amounts of impurities by physical or immunochemical methods. The explanation resides in the finding that preparations of crystalline papain or mercuripapain undergo an irreversible inactivation on storage which results in a parallel loss of sulfhydryl which is reactive with PCMB. Indeed, because this change takes place in crude latex, different batches of crystalline papain show a variable activity, a phenomenon first noted by Balls and Lineweaver (7).

The variation of activity with reactive SH supplies strong, additional evidence that the SH group must be directly concerned in the activity of the enzyme.

More limited studies with iodoacetamide also show that less than one equivalent of this substance is bound by the enzyme, and that this produces complete inhibition (6).

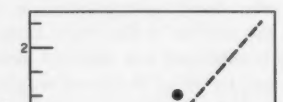


FIG. 1. Relationship between proteolytic activity (C_1) towards benzoyl-L-argininamide and SH reactive towards *p*-chloromercuribenzoate as estimated by a spectrophotometric method (Finkle and Smith, unpublished).

The results obtained by study of the papain reaction with mercury, PCMB or iodoacetamide are consonant with the view that there is only one reactive SH group in the enzyme and that this group must be a part of the active site. The broad specificity of papain has also posed the problem as to whether a single 'active center' is responsible for the action on all types of substrates. The observation that carbobenzoxy-L-glutamic acid inhibits the hydrolysis of such diverse substrates as benzoyl-L-argininamide, hippurylamide, and carbobenzoxy-L-isoglutamine is in agreement with other evidence that only one active center is responsible for the different catalytic activities (2).

Although the above studies indicate that only one sulfhydryl group is concerned in the activity, it is essential to determine the total number of such groups in the molecule. Preparations of papain denatured in various ways have been found to react with more than 1 mole of PCMB. Under some conditions, papain will react with as many as 6 moles of PCMB, thus showing that there are 6 SH groups in the molecule (4). It has been found by analyses of hydrolysates of oxidized papain that the molecule yields 6 moles of cysteic acid (8). The agreement between these results suggests that cystine itself is absent from papain and that 6 moles of cysteine are present. However, in the native protein, under ordinary conditions, 5 of the SH groups do not react with PCMB.

STUDIES OF STRUCTURE

The aforementioned studies suggest that only one of the 6 SH groups of papain is concerned in the activity, and we shall proceed on the assumption that in the native protein, the SH group which reacts with mercury, PCMB or iodoacetamide is uniquely concerned in the activity. One of our problems is then to determine the location of this SH group in the protein and to establish the structure of the protein in the vicinity of this reactive SH group. There are a number of possible approaches to the study of the structure of a protein molecule as large as that of papain and, in our laboratory, we are using several of the methods which have been successful in the studies of other large polypeptides and proteins.

Using the dinitrophenyl amino end group

method of Sanger (9), Thompson (10) demonstrated that papain contains only one free α-amino group, that of isoleucine and that there is a unique N-terminal tripeptide sequence isoleucylprolylglutamyl . . . . These results indicate that papain consists of only a single peptide chain. Since amino acid analysis indicates the presence of about 180 residues (11), the remaining task is to allocate the other 177 residues.

The main procedures which have been used in the past years for analysis of amino acid sequences in large polypeptides and proteins depend on treatment with performic acid to oxidize disulfide bonds and sulfhydryl groups, followed by some type of partial hydrolysis. Partial hydrolysis by acid yields mainly di- and tripeptides and has been useful mainly with smaller polypeptides or for identification of peptides containing certain types of residues. Because of the importance of the cysteine residues, a study was made of partial acid hydrolysates of papain which had been oxidized with performic acid (8). The oxidized protein was treated for 7 days in 12 N HCl at 37° and the hydrolysate worked up in the usual manner. The strongly acidic fraction containing cysteic acid and its peptides was separated by electrophoresis on paper in acetic acid at pH 2.3, or by passing the hydrolysate through Dowex-50-X4 in the hydrogen cycle, the acidic fraction emerging unretarded from the column under these conditions. In both cases, peptides were separated by two-dimensional paper chromatography. The peptides obtained by this procedure, whose structure has been completely or partly determined, are listed in table 1. Obviously we have not identified all of the sequences involving cysteic acid in the oxidized protein, possibly because we attempted to isolate only the acidic peptides. Neutral peptides, e.g., those containing cysteic acid and a basic residue, would not have been detected by the methods which were used.

Perhaps the only valid comment that can be made at this point is how singularly uninformative these results are. It is obvious that there is nothing unique about these sequences and they yield no clue as to which one, if any, is involved

TABLE 1. CYSTEIC ACID PEPTIDES DEFINITELY IDENTIFIED FROM PARTIAL HYDROLYSATES OF OXIDIZED PAPAIN (8)

Asp·$CySO_3H$	$CySO_3H$·Asp
Ser·$CySO_3H$	$CySO_3H$·Gly
Val·$CySO_3H$	$CySO_3$·Gly·Asp
Gly (Pro, $CySO_3H$)	

TABLE 2. AMINO ACID COMPOSITION OF PAPAIN (11, 8)

Amino acid	Residues	Amino acid	Residues
Asp	17	Leu	9
Thr	7	Tyr	17
Ser	11	Phe	4
Glu	17	His	1
Pro	9	Lys	8
Gly	23	Arg	9
Ala	13	Tryp	5
Val	15	½ Cys	6
Ileu	9	Amide	(19)
		Total	180

in the enzymic activity. It is also apparent that isolation of small peptides is not a very fruitful procedure with a protein as large as the papain molecule. Therefore, several years ago we turned to the use of proteolytic enzymes for partial hydrolysis of oxidized papain (12, 13). This approach has, of course, been very useful for structural studies of many other proteins and large polypeptides.

The amino acid composition of papain (11), shown in table 2, reveals only a few unusual features. These may be mentioned briefly as the absence of methionine, the presence of only 1 histidine and 4 phenylalanine residues and the rather high content of tyrosine and glycine. Papain contains 8 lysine and 9 arginine residues. Inasmuch as trypsin can hydrolyze only peptide bonds involving the carboxyl groups of these basic residues, it may be expected that this enzyme will hydrolyze 17 bonds unless arginine or lysine is C-terminal in the protein. Moreover, each peptide obtained by tryptic digestion should contain C-terminal lysine or arginine except possibly the C-terminal sequence.

Mercuripapain was oxidized with preformed performic acid and the excess performic acid was removed by freeze-drying. Control studies have demonstrated that no detectable cleavage of the peptide bonds of papain is produced by this treatment (8). The oxidized protein was suspended at pH 7.0–7.5 and digested with crystalline trypsin (12). The resulting peptides were separated by chromatography on a column of Dowex-50-X2 in the sodium form essentially as described by Hirs, Moore and Stein (14). A system of buffers of increasing ionic strength and pH was used for the resolution of the components which reacted with ninhydrin. Fractions were collected with the aid of an automatic fraction collector. Some of this work has already been presented (12, 13).

There is no point, at present, in describing in

detail our incomplete studies of these peptides but the general approach may be indicated. Each peptide has been hydrolyzed and then analyzed by the chromatographic method of Moore and Stein (15). Purity of each fraction has been judged not only by the amino acid analysis but also by paper electrophoresis and by end group studies.

One peptide obtained from the tryptic hydrolysis is of special interest. This is Peptide 1 which emerges at the column volume (12), and is, as might have been anticipated, a strongly acidic substance. Quantitative analysis of this peptide gave the following composition: [Cys_2, Asp_2, Thr_1, Ser_2, Glu_3, Pro_1, Gly_5, Ala_1, Val_2, $Ileu_1$, Leu_3, Tyr_4]. The substance contains 27 residues and lacks lysine or arginine. Since it is only the peptide which has been obtained from the tryptic digest which does not contain a basic residue, it would seem to be the C-terminal portion of the papain molecule.

A feature of special interest is the presence of 2 residues of cysteic acid. Since these are derived from cysteine and represent 2 of the 6 residues of this amino acid, there is a fair chance that the C-terminal end of the papain molecule contains the active center. It may be recalled that our choice of papain as an enzyme for structural investigation rests on the hope that we can identify the active site because of the presence of an essential sulfhydryl group.

It should be noted that the yield of Peptide 1 has been only modest (10–25%) and in some cases has not been detected. This was somewhat puzzling until the acid-insoluble fraction which accumulates during the tryptic digestion was investigated. One peptide, purified by paper electrophoreses, proved to represent a large portion of the papain molecule since, by analysis, it contained more than 50 residues. What is striking is that the amino acids of Peptide 1 are present in this insoluble peptide (Peptide In-1) and that only one basic residue, lysine, is found in the material. Our present supposition is that Peptide In-1 may represent the C-terminal 50 odd residues of oxidized papain and that the single lysine residue is linked to the N-terminal end of Peptide 1 in a bond which is only poorly susceptible to tryptic digestion.

A great deal of work has already been done with the peptides obtained from oxidized papain by tryptic digestion. Obviously, it will require considerably more effort to complete this phase of the investigation. In addition, other methods of hydrolysis will be needed to permit fitting these sequences together. The methods are available but progress is necessarily slow with a protein as large and complex as papain. Even Peptide 1, with 27 residues, is almost the same size as glucagon (16) and as the B-chain of insulin (17). Yet this 27 residue sequence is only about one-seventh of the papain molecule.

DEGRADATION BY LEUCINE AMINOPEPTIDASE

Fortunately, other approaches to the structure of papain are available, in particular the use of highly purified leucine aminopeptidase (18) for successive liberation of residues from the amino terminal end of the molecule. It has been established with synthetic compounds (19), as well as with larger polypeptides, that the aminopeptidase can attack only the peptide bond adjacent to a free α-amino group (20). The enzyme has been successfully used, for example, with the oxidized B-chain of insulin to determine the sequence of the first 6 residues at the N-terminal end of this polypeptide (20).

Papain itself cannot be used as a substrate for the aminopeptidase since the aminopeptidase is rapidly inactivated by this proteinase (21). However, mercuripapain is essentially inert. As previously reported (22–24), the aminopeptidase can liberate a considerable quantity of amino acids in 24 hr., the actual extent of hydrolysis depending on the ratio of enzyme to substrate (24). What is most important is that after as many as two-thirds of the amino acid residues of mercuripapain have been liberated, the enzyme still retains its activity. This has been tested after removal of mercury from the undegraded fragments of mercuripapain. These results indicate that the active site of the papain molecule is remote from the N-terminal region of the protein.

Analyses for the amino acids liberated at several stages of digestion with the aminopeptidase have been performed (21). It is noteworthy that at no point does the relative composition resemble that of intact papain, or for that matter of the aminopeptidase. Thus, these data show that the composition of the N-terminal part of the papain molecule is different from that of the C-terminal region. In particular, most of the threonine, alanine, leucine and isoleucine are in the first 73 residues whereas most of the tyrosine and valine appear to be in the remainder

of the molecule. It is interesting that only 1 of the 6 cysteine residues has been definitely located in the N-terminal region. Obviously this cannot be the one concerned in the enzymic activity.

The dinitrophenyl method reveals only α-DNP-isoleucine and 8 moles of ε-DNP-lysine in intact papain (10). After 19 residues have been liberated by the aminopeptidase, no DNP-isoleucine could be detected (24). The DNP method yielded mainly DNP-arginine with smaller amounts of DNP-alanine and DNP-aspartic acid. Moreover, only 7 moles of ε-DNP lysine could be obtained but since 1 mole of lysine was liberated, the total of 8 residues checks with the amount of lysine in the intact protein. After an apparent liberation of 39 residues by the LAP, the residual mercuripapain was again studied by the DNP method. No DNP-isoleucine the original N-terminal residue, or DNP-arginine, the predominant end group after removal of 19 residues, could be found. Instead, large amounts of DNP-valine, DNP-aspartic acid and smaller amounts of the DNP derivatives of serine, alanine and glutamic acid were found. A mixture of end groups is to be expected, since the enzymic degradation by LAP proceeds stepwise and independently in each molecule of the substate, mercuripapain. The studies of the liberated amino acids and of the end groups in the undegraded portions of the mercuripapain are all in accord with the view that the aminopeptidase acts only on the N-terminal end of the molecule.

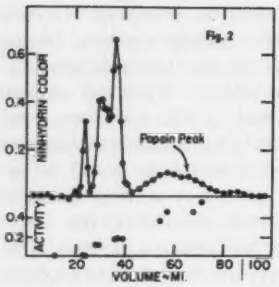
The finding that a considerable part of the papain molecule is unessential for activity is obviously of general interest. Tests made with synthetic substrates of rather different structure show that the relative specificity of papain does not change on degradation. Such studies have been made with benzoyl-L-argininamide, carbobenzoxy-L-glutamic acid diamide, hippurylamide and carbobenzoxy-L-leucinamide (21, 24).

Recently, confirmation that partially degraded papain can retain its proteolytic activity has been obtained by another method (25). Crystalline papain can be successfully chromatographed on IRC-50 in neutral or slightly acidic phosphate buffer. At pH 7.0, the protein appears to be essentially homogeneous. At pH 6.2, the protein shows a major active peak and very small amounts of inactive material. On the assumption that at least part of the inactive material represents products of autolysis, a solution of active papain was allowed to undergo autolysis. During this procedure the enzyme lost approximately 50% of its original activity. When this solution was chromatographed, it was found that the original active peak had diminished in quantity and other material appeared, as would be expected (fig. 2). What is very striking, however, is that a considerable portion of the active material emerged at elution volumes very different from that of intact papain. As might be expected, the yield of such active fragments was small since active papain can digest other molecules at a variety of sites which cannot be controlled. Inasmuch as the results with LAP digestion indicate that the active site is not at the N-terminal region of the papain molecule, it may be assumed that an autolytic cleavage at the N-terminal end will produce active, modified papain molecules whereas a primary, autolytic cleavage at or near the C-terminal region will produce inactive fragments. Actually most of the fragments resulting from the autolysis appear to be inactive.

The results obtained by LAP digestion and by autolysis offer the hope that it may not be necessary to determine the complete structure of the papain molecule in order to understand its specificity and mechanism of action. This task could possibly be accomplished by a determination of a limited part of the structure.

LABELING OF ACTIVE SITE

Inasmuch as iodoacetamide (IAA) reacts irreversibly only with the active sulfhydryl group of papain, an attempt has been made to label the active site with this reagent. Reduced papain was treated with one equivalent of IAA and then dialyzed exhaustively. The treated protein was then oxidized with performic acid in the usual way, digested with trypsin and the material chromatographed in the same manner as the digest of untreated papain. Peptides known to contain cysteic acid were hydrolyzed with 6N HCl and examined by a paper chromatographic method. Only the insoluble fraction (Peptide In-1) contained material which migrated at the same rate as authentic carboxymethyl cysteine sulfone (25). This finding indicates that IAA does not react at random with all the SH groups of papain and that it is likely that its reaction is limited to the specific SH group of



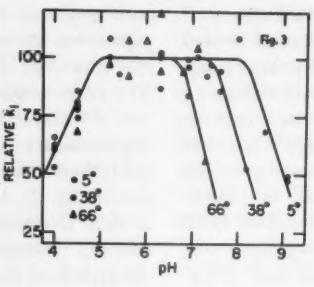


FIG. 2. Chromatography of partially autolyzed papain on IRC-50 in 0.2 M phosphate buffer at pH 6.2. Untreated papain shows a nearly symmetrical peak at the position indicated with only a small amount of slightly faster moving material. In the autolyzed sample it is evident that activity (given in arbitrary units) is associated with several peaks (Finkle and Smith, unpublished).

FIG. 3. Effect of pH on k_1 for the hydrolysis of benzoyl-L-argininamide. The average k_1 for the broad maximum was set equal to 100 for each temperature. From (28).

the active site. It should be possible, therefore, to isolate and identify the region of the papain molecule which bears the reactive SH group.

KINETIC STUDIES

It should be emphasized, however, that we do not feel that a determination of amino acid structure by itself will offer a satisfactory understanding of the action of this or any other enzyme. We must be able, eventually, to correlate the structure of the enzyme with its specificity and its kinetic behavior towards a rather large variety of synthetic substrates. For this reason, detailed kinetic studies have been undertaken with a variety of substrates. Only one aspect of these studies will be mentioned here since they have a bearing on the structure of the active site.

The familiar formulation of an enzyme reaction is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_0} P + E$$

where E is enzyme, S substrate, P products and ES the enzyme-substrate complex. By conventional methods we may determine k_0 and K_m. However, K_m is a complex constant which is $(k_0 + k_{-1})/k_1$. It has been shown for some enzymes that $k_0 \gg k_{-1}$ (26, 27). In such cases, $K_m = k_0/k_1$.

Considerable evidence has been obtained that this is the situation for papain (28). Thus, by determining K_m and k_0, we can calculate the value of k_1, the velocity constant for the formation of the ES complex. This has been done over a broad pH range at three temperatures for the hydrolysis of benzoyl-L-argininamide. For the present, let us consider only the calculated values of k_1. Figure 3 shows these data with the k_1 values arbitrarily superimposed at their maxima. It is striking that all of the data have the same general form at the acid pH values but the descending alkaline side of these curves has been displaced at the different temperatures.

The simplest assumption which can be made to explain these data is that the descending portions of the curves represent the titration of groups essential for the k_1 step. The pH corresponding to k_1 at half of the maximal value is near pH 3.9, which would represent the pK′ of the group being titrated. This pK′ value is consistent with that of a carboxyl group. The lack of any appreciable effect of temperature on this value is in agreement with the knowledge that the heat of ionization of a carboxyl group is small (29).

The alkaline portions of the k_1 curves may be interpreted in a similar manner but it is evident that pK′ of the group being titrated varies markedly with temperature. The apparent heat of ionization is approximately 9.5 Cal/mole at 0°. Titratable groups which, at first sight might be involved are an α or ϵ-ammonium group, an imidazolium, a phenolic or a sulfhydryl group. It is very unlikely that the strongly cationic substrate, benzoylargininamide, could interact with a cationic site on the enzyme. Moreover, the calculated pK′ values are much too low for an ϵ-ammonium or a phenolic group, and too high for an imidazolium group. Papain possesses only one α-amino group but, as already emphasized, much of the N-terminal sequence can be removed without impairing the activity of the enzyme. This would suggest that the α-amino group does not participate in determining the value of k_1.

In effect, the only remaining group to be considered is the sulfhydryl group, and we already

know that such a group is concerned in papain activity. For the present, it may be noted that the pK′ values are in the range recently found for sulfhydryl groups (30) but the calculated heat of ionization appears to be rather high. However, these data are rather limited and the precision does not permit very precise comparison. For this reason we have studied the effect of pH on the hydrolysis of other substrates and, now knowing the important range to be studied, more complete data have been obtained.

Figure 4 shows the effect of pH on k_1 for the hydrolysis of carbobenzoxy-L-histidinamide (31). k_1 has been calculated in the same manner as previously mentioned. The data have been fitted by drawing theoretical titration curves for a single titratable group at both sides of the curve. On the acid side of the curve, there is little effect of temperature and the apparent pK′ at 38° is near 4.3. On the alkaline side, there is a shift of pK′ with temperature and the calculated ΔH, the heat of ionization, is about 4.4 Cal/mole at 0°. Both the pK′ value and the value of ΔH are near those expected for a sulfhydryl group (30).

The effect of pH on k_1 for another type of substrate, carbobenzoxyglycylglycine, is shown in figure 5 (31). The curve on the alkaline side has been fitted by a theoretical titration curve with a pK′ near 8.0 which is close to that found for the other substrates at 38°. However, there is a plateau near pH 6 and k_1 rises sharply at more acid pH values. It should be noted that at pH 6,

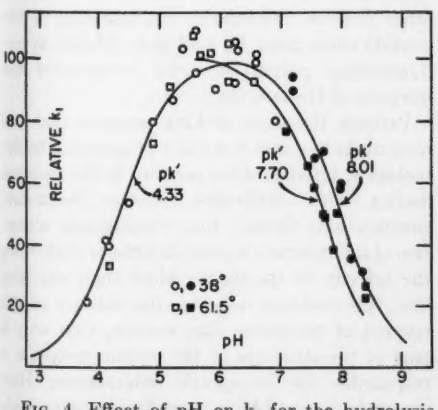


FIG. 4. Effect of pH on k_1 for the hydrolysis of carbobenzoxy-L-histidinamide. The maximum of each temperature was set equal to 100. From (31).

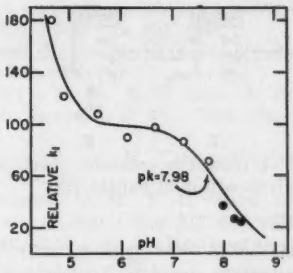


FIG. 5. Effect of pH on k_1 for the hydrolysis of carbobenzoxyglycylglycine at 38°. From (31).

the absolute value for the k_1 of carbobenzoxyglycylglycine is only about 0.1% of that for benzoylargininamide. At pH 6, both the enzyme and carbobenzoxyglycylglycine have ionized carboxyl groups and electrostatic repulsion may be expected. The rise in k_1 values below pH 5.5 appears to be due to repression of ionization of one or both carboxyl groups, thus decreasing the degree of repulsion between enzyme and substrate and, in effect, producing an increase in the value of k_1.

All of these kinetic data point to the participation of the essential sulfhydryl group in the k_1 step. Moreover, since the optimal region is near pH 6, these data indicate that the active form of this group is RSH and not RS^-. In addition, evidence has been obtained that an ionized carboxyl group participates in the k_1 step. Inasmuch as the substrates which have been used are rather small, this carboxyl group must be close to the SH group. The simplest, but not the only possible assumption is that this is on a residue adjacent to the active SH group. If so, the carboxyl group must be supplied by an aspartyl residue. It will be recalled that peptides were isolated from oxidized papain which have the structure, aspartyl cysteic acid and cysteylaspartic acid (table 1). No acidic peptides containing cysteic and glutamic acids were found and such peptides would have been readily detected by the methods which were used.

A TENTATIVE REACTION MECHANISM

Thus, from kinetic and other studies, the evidence points to the participation of the sulfhydryl group in the enzyme-substrate interaction and to an important role of an ionized carboxyl group in this reaction. A tentative reaction mechanism for the action of papain has been suggested (4, 28). The main feature of this

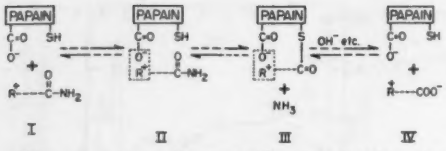


FIG. 6. A tentative reaction mechanism for the hydrolytic action of papain (28).

scheme is that a thiol ester is formed in the enzyme-substrate complex. A schematic outline of the proposed reaction steps for the hydrolysis of BAA is shown in figure 6.

Briefly, the following observations must be considered. *1*) The most sensitive known substrates for papain are those which possess an arginyl or lysyl residue; *i.e.*, they have a strongly cationic group on the side chain. *2*) Papain can hydrolyze synthetic substrates which possess ester (2) or thiol ester (32) bonds, as well as those with peptide or amide bonds. *3*) In addition to hydrolytic reactions, papain catalyzes synthetic and transfer reactions (1). *4*) There is one sulfhydryl group which is absolutely essential for the action of papain (4, 6).

If it is assumed that the rate-limiting step governed by k_1 represents the formation of a thiol ester bond, reasonable explanations for the effect of pH on k_1 are apparent. The presence of an essential ionized β-carboxyl group in the enzyme and of a cationic group in the substrates which are hydrolyzed most readily by papain suggests that an ionic interaction between enzyme and substrate (II in fig. 6) enhances the rate of formation of ES. Formation of a thiol ester (III in fig. 6) is postulated because of the essential nature of the SH group and because thiol esters appear to be formed in other types of enzyme reactions (33).

Formation of a thiol ester is consistent with the spatial and chemical specificity of papain which is toward the amino acid residue bearing the carbonyl group of the sensitive peptide bond. The moiety which is displaced from or transferred to the carbon of this carbonyl group may be any of a large variety of compounds, e.g., a peptide, an amino acid, ammonia, aniline, hydrazine, hydroxylamine, an alcohol, a thiol, etc. (1).

Postulation of an intermediate such as III is necessary to explain the efficiency of papain in catalyzing transfer reactions since the replacement reagent R—NH_2 is competing with water or the ions of water for the hydrolytic reaction. The expected free energy change for the hydrolysis of the thiol ester bond would indicate that the equilibrium would be greatly in favor of hydrolysis.

It should be emphasized that in the reaction scheme of figure 6, there has been no attempt to explain all the features of papain specificity nor to indicate the total number of steps from I to IV. Indeed, it is likely that other groups on the substrate may also interact with the enzyme. Postulation of a three point interaction may be necessary to explain the optical specificity of papain. For the present, the scheme of figure 6 may be regarded as a minimal, working hypothesis.

CONCLUDING REMARKS

It is evident from the foregoing summary of our investigations of papain that something has been learned, but that much more remains to be done. In concluding there are perhaps just a few points which remain to be emphasized. Among them is the obvious one that we shall not understand the mechanism of enzyme action only by studying kinetics or substrate specificity, or only by determining amino acid sequences. All such studies are necessary and the information must be correlated. Second of all, amino acid sequences will not yield a complete understanding of the structure of the active site of an enzyme like papain. The fact that papain can be denatured by heating, although it is more stable than most enzymes, indicates that the specific activity depends on more than the primary structure determined by amino acid sequence. As with other proteins, the specific configuration of the peptide chain must be held in a definite, three-dimensional pattern in order to preserve the integrity of the enzyme.

Perhaps the most striking progress that has been made in recent years in our approach to the biological activity of the proteins is the common finding with antibodies, protein hormones, enzymes and viruses, that considerable alteration of the structure is possible without destroying the activity or specificity. More than anything else, this evidence indicates the validity of the concept of the active site, namely, that only a part of the structure of the protein molecule is responsible for its specific interactions. How successful we shall be in degrading proteins without destroying activity will depend in part on the tools at hand. For the moment the best of these reagents appear to be the proteolytic enzymes,

among them leucine aminopeptidase, but we need more such tools and also a little luck.

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DEGRADATION AND STRUCTURE OF TOBACCO MOSAIC VIRUS

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TOBACCO mosaic virus is a rod shaped particle, 300 mμ long, which is composed of approximately 94% of protein and 6% of nucleic acid. The protein forms the outer shell around the nucleic acid cylinder. Recent studies have clearly demonstrated that the nucleic acid alone can initiate infection and transfer all the necessary 'information' for the production of complete virus (1–3). The efforts of virologists are, therefore, now centering on this crucial minority constituent of the virus. However, the role played by the protein moiety in various important regards must not be overlooked. In quantitative respects, the protein contributes to viral infectivity, since it potentiates the action of the nucleic acid by 20- to 50-fold, possibly by giving it protection and rigidity (4, 5). The protein also accounts for the serological activities of viruses which are of such practical importance, and the protein probably influences host range specificities and virulence. Furthermore, the protein components have been shown to differ in specific and characteristic manner from one virus strain to another (6, 7), and may, therefore, be regarded as phenotypic expressions of the chemically still obscure genotypic characteristics of viruses. This symposium has clearly documented our advances towards the goal of complete protein structural analysis. The possibility exists, therefore, that the structure of the virus protein may be elucidated within the next few years. The virus may then become the Rosetta stone of biochemical genetics, in supplying the bilingual record needed to decipher nucleic acid in terms of protein structure.

While the protein of one virus particle weighs as much as 40–50 $\times$ 10^6 hydrogen atoms, it has been recognized for several years that this represents an aggregate of much smaller protein subunits or molecules. The minimal molecular weight, based on amino acid analyses, is about 18,000. One cysteine and two lysine residues occur in all strains of TMV, and one histidine in the H.R. strain, in about 18,000 gram moles (4, 6, 7). That this approximates the true molecular weight of the smallest unit was strongly suggested by the discovery of Harris and Knight that carboxypeptidase releases 2700 threonine residues—no more, and no other amino acids—from each virus particle, or one residue per 17,300 (8, 9). It has since been established beyond doubt that this represents a C-terminal group (10, 11), and that the virus is actually composed of about 2700 separate peptide chains, held together only by secondary forces. Since 'macro-structure' is the main function of this protein, it is not surprising that those secondary forces are much in evidence, so that the subunits are hard to separate, and even harder to keep separated under conditions favorable for physicochemical characterization. Thus, only rough approximations of the subunit weight have as yet been made on the basis of sedimentation studies (12), but these support the concept that single peptide chains supply the bricks building up the super-molecule.

Degradation of Virus and Isolation of Protein. A variety of methods have been used for the degradation of the intact virus and for the isolation of disaggregated nucleic acid-free protein. Detergents, such as sodium dodecyl sulfate (SDS), represent the most efficient agents for the disruption of all interunit bonds, but the protein obtained from such reaction mixtures shows many undesirable features, some of which will be referred to later. It appears that these agents break not only inter-chain but also intra-chain secondary bonds. Thus the product is not the expected mixture of sticky bricks, i.e., subunits of definite shape, but a mixture of sticky threads, i.e., peptide chains, and the result is a tendency to unlimited random aggregation into a most untractably insoluble material.

Gentler methods for the isolation of the protein are based on a procedure first proposed by Schramm *et al.* (13) in which the virus is solubilized by dilute alkali (about pH 10.5) in the cold. Carbonate, glycine, and more recently alkanolamine buffers have been used for this purpose (14). Harrington and Schachman have recently studied the kinetics of this degradation, and demonstrated the great tendency for reaggregation of split products in the alkaline reaction mixtures (15). Ammonium sulfate can conveniently be employed to separate the protein cleanly from the nucleic acid after all types of degradation. The final protein preparation obtained from alkali-degraded virus appears 'na-

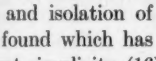
tive' in various respects, to be discussed below, and gives water-clear solutions except in the isoelectric region (pH 3.5–6.5). In that pH range there occurs a remarkable reaction: The solution becomes opalescent and its 'virus-like' appearance is borne out by electron micrographs which show that most of the material is again in the form of rods of great variability in length, but exactly the same diameter as the original virus. Thus here we are dealing with a solution of building blocks 'with memory' or, in less anthropomorphic terms—the biological activity of this particular protein, namely its tendency to controlled aggregation resulting in a particular macro-structure, has not been destroyed in the course of degradation. The sedimentation coefficient of this material in solution (usually determined at about pH 9) resembles that of Schramm's electrophoretically separated A-protein (about 4S) and suggests a molecular weight of about 100,000 corresponding to six-chain aggregates. It appears quite possible but by no means proven that units of such a size are required for the proper functioning of this protein.

A convenient method for the breakdown of the virus and isolation of the protein has recently been found which has the particular advantage of great simplicity (16). The virus is exposed to cold 67% acetic acid, the precipitate which flocculates in a few minutes is centrifuged off (largely nucleic acid), and the protein which alone remains in the water-clear solution is isolated after dialysis. This type of protein preparation appears indistinguishable in all respects from that obtained by alkali splitting and ammonium sulfate fractionation. This is at present the method of choice in our laboratory.

Tertiary Structure of Virus Protein. From the preceding description it is clear that the protein as isolated from dilute alkali or from acetic acid must retain a definite shape which enables it to bridge the gulf between a 100,000 molecular weight protein and a 50 million particle weight virus rod. What type of bonds are involved in maintaining its structure? This question has repeatedly been attacked in our laboratory. One amino acid side chain which is very probably implicated in this structure is the cysteine-SH group. This group was found to exist in a form which protects it from many —SH reagents of various types, including oxygen, and thus renders it as stable as the masked —SH groups of many other proteins (17). On the other hand the —SH group can readily be titrated after unmasking it by the usual denaturing agents (such as guanidine HBr), and represents a convenient means of estimating to what extent a protein preparation has retained its original native character. It was found to be quantitatively present (one per 18,000) in the two types of soluble protein described above (16), but absent, presumably autoxidized, in the detergent type of preparation.

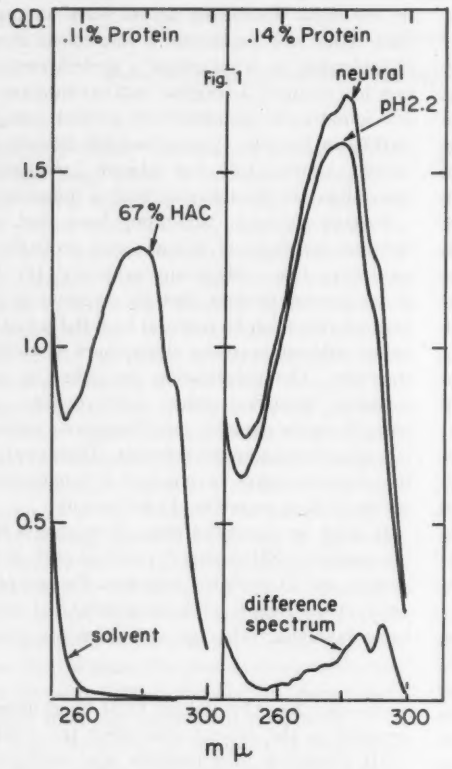
Further studies of this group have shed new light on its mode of linkage, and probably of masked protein —SH groups generally (18). For it has become evident that the H atom of this masked group can be replaced by CH_3Hg and by iodine without apparent disturbance of protein structure. The substitution by iodine is particularly surprising since sulfenyliodides are normally quite unstable and decompose within a few seconds in aqueous solvents. This occurs in the virus derivative as soon as it is denatured by agents such as guanidine hydrobromide.

It must be concluded from these results that the masked —SH groups *1)* occur as such in the protein and *2)* probably represent the acceptors rather than donors of hydrogen-bonded structures involving other as yet unknown groups



(—S----H—X). This linkage must be of unusual firmness in the present case since the masked —SH groups of other proteins, e.g., ovalbumin, do not give stable sulfenyliodide derivatives, although their reactivity otherwise resembles that of TMV.

Another type of tertiary bond, which is at present receiving much attention from the protein chemists, is the hydrogen-bonded interaction of the phenolic groups of tyrosine residues with the carboxylate ion groups of glutamic or aspartic acid residues. Evidence for the presence of such bonds is often based on the spectral shift to longer wave lengths observed for tyrosine so bonded. If this spectral shift is largely reversed in acid solution, and if the pK of this effect is within the range of that of carboxyl groups, then a tyrosine-carboxylate interaction is postulated (19, 20). In the case of the native TMV protein the spectral divergence from that of an *ad hoc* prepared amino acid mixture has already been pointed out (4) as well as the correction of this divergence by the use of an acidic solvent. Difference spectra have now been plotted for the protein at pH 7 as compared to pH 2.2, and these were found to resemble closely those obtained with ribonuclease, insulin, etc. (see fig. 1). Thus



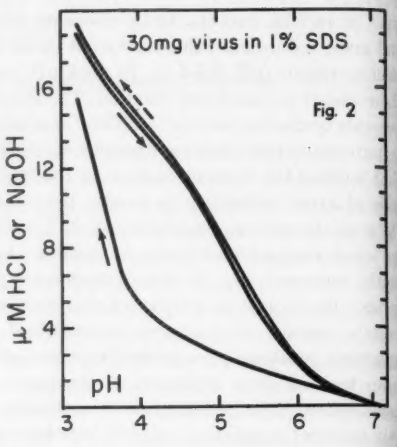
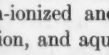


FIG. 1. UV absorption of TMV-protein in 67% acetic acid, pH 2.2 HCl, and water.
FIG. 2. Alkali consumption during degradation of TMV by 1% sodium dodecyl sulfate below pH 4, as indicated by back titration to original pH.

it appears probable that bonds of the tyrosine-carboxylate type do occur in the isolated virus protein. It must be noted, however, that the making and breaking of these bonds appears readily reversible and that they are not stable under some, if any, of the conditions required for the degradation of the virus prior to isolation of the protein. Thus it would appear impossible to attribute to these bonds any major role in maintaining the original folding and shape of the protein. Nor can they play part in the inter-unit bonding, unless originally intermolecular bonds of this type are presumed to have exchanged partners during degradation of the virus to give the possibly weaker intra-molecular bonds observed in the final preparation. A search for spectral differences between the intact virus and that degraded by acid (+SDS) require correction for the light scattering by the intact virus. Comparison between the acid and pH 8 SDS degraded virus need no such correction. In both cases a shift in the opposite direction from that given by the protein alone was obtained, the acid solution giving the positive difference spectrum with a maximum about 290 mμ. However, virus nucleic acid was found to show a similar behavior, which makes such reaction mixture too complex to attempt any interpretation of the spectral changes.

Nature of Inter-Unit Bonds. Previous studies by Harrington, Levy and Schachman (21) have indicated that OH^- ions are rapidly consumed when TMV is degraded in weakly alkaline solutions. These studies have now been extended to a new range of experimental conditions for the splitting of the virus. For it has been observed that the virus in the presence of 1% SDS loses its opalescence rapidly at room temperature when exposed to pH 3.5. (Nucleic acid of similar infectiousness as the standard preparations can be isolated from such reaction mixtures, as well as nucleic acid-free, though denatured protein.) When the reaction mixture containing the degraded virus was subsequently titrated back to the original pH (usually pH 7.0), more alkali was required than that equivalent to the acid used to

reach the pH at which the virus was degraded. The difference was similar to the alkali consumption observed in the earlier experiments (21), and corresponded to about 1.5 μ M/18 mg virus. Subsequent titrations between pH 7.0 and 3.5 required equivalent amounts of acid and alkali, and followed the same curve as the first back titration (see fig. 2). A working hypothesis which could account for these observations is as follows: A certain number of carboxyl groups of the virus (about 4000) are hydrogen bonded in the undissociated $-C(OH)=O$ form to some other, possibly tyrosine, groups. This bond is so stable as to prevent the carboxyl groups from dissociating until disaggregation of the macro-molecule re-establishes their normal pK and titrability. Carboxyl-tyrosine bonds have been proposed by Edelhoch for pepsin (22), and are in that case believed to be stable up to about pH 6, at which point pepsin becomes denatured. In the case of TMV one would have to assume an extension of the pH stability range of such bonds to almost pH 9. Tyrosine-carboxyl (undissociated) interactions have been observed in model experiments of Laskowski (23), and there are indications that they may produce much smaller spectral shifts than does the carboxylate-tyrosine interaction. However, such an effective masking of protein carboxyl groups has not previously been demonstrated and will have to be supported by much more experimental evidence than is available at present. One alternate possibility which could account for the presented observations is that about 4000 secondary phosphate —OH groups ($-R-O-P(=O)-O-$), rather than carboxyl groups,



are un-ionized and involved in structural stabilization, and aquire their normal dissociability only upon degradation of the virus. Unusual bonds of some kind or other will probably be required to explain the unusual stability of the intact virus as compared to its major component, the protein. Simple aggregation, as observed with so many proteins, does not generally evoke major changes in character. Yet the virus is stable at temperatures above 60°, while the isolated protein becomes denatured below 40° (24). Differences in resistance towards enzymes and other agents may be expressions of the same phenomenon. The fact that the ease of degradation of the virus differs for different strains, as well as for chemical derivatives of the same strain, also points to the existence of a definite determining and limiting factor in the inter-unit bonding. It has also been noted that the electrophoretic mobilities of the virus, as well as of various virus protein aggregates are often higher i.e., the protein less acidic than one would except from the amino acid composition. An unexplained shift in the opposite direction was observed for the enzymatically dethreoninated virus (8). Preliminary experiments with our titration technique suggests that the latter contains fewer 'masked' carboxyl groups than the original virus. It would appear that carboxyl-masking could be a determining factor in the observed electrophoretic differences, as well as in immunological and other particle surface properties.

Studies of the Primary Structure of TMV. During the past years various studies have been initiated by several investigators in the Virus Laboratory to solve structural and peptide sequence problems connected with the TMV protein. The steps involved are, *1*) enzymatic degradation of the protein; *2*) separation of pure peptide fragments; *3*) elucidation of their composition and sequence; and *4*) fitting together of the pieces on the basis of overlaps supplied by the two or more sets of peptides obtained through the use of two or more different enzymes.

1) Considerable effort has been made to use the SDS-denatured protein, which is a by-product of the nucleic acid preparation, for these studies. However, the susceptibility of this material to enzymatic digestion has been found to be somewhat erratic. Digestion of the acetic-acid prepared protein with 1% trypsin (Worthington, cryst.) at 37°, i.e., near its point of heat denaturation for about 8 hr. has given the most reproducible results. The ninhydrin analyses indicate the splitting of, maximally, about 12 bonds (leucine equivalents). The C-terminal groups found in hydrazinolysis experiments by Dr. Narita in the acid-soluble fraction of the digest were, besides the original C-terminal threonine, 9 arginine, 1.3 lysine, and about 0.25 residues of leucine and serine. This indicates a high degree of specificity for the enzyme used, but falls slightly short of quantitative digestion (expected: 11 arginine, 2 lysine groups cf. ref. 25), although the high correction factors, particularly for arginine, lessen the strictly quantitative significance of these values. The N-terminal groups found (Niu, Ramachandran) are about one each of aspartic and glutamic acid, serine, threonine, phenyl-

alanine, and tyrosine, possibly two of glycine, and less than one of alanine, valine and leucine, in quantitative accord with the number of C-terminal residues.

Chymotrypsin digestion liberated about 15 leucine equivalents. C-terminal residues were (besides the original threonine) phenylalanine (6 residues), leucine and isoleucine (3), tyrosine (3), tryptophane (about 1), (total of these amino acids: 8, 10 + 6, 4 and 3, ref 25) and traces (<0.2) of serine, glycine, valine and proline. The N-terminal residues found in such digests were aspartic and glutamic acid (about 3 and 2), valine (2), and about one each of threonine, serine, leucine and phenylalanine.

2) For separation of the peptides we have used Dowex 50-X-2 columns as advocated by Moore and Stein (26) with some success. Several sharp peaks, however, could be resolved into two or three components by subsequent paper chromatography. At present Dr. D. Gish is subjecting the digests to countercurrent distribution and appears to get good resolution of the mixture, although here also seemingly homogeneous peaks may occasionally contain more than one component. Dr. Knight and Miss Woody have employed a two dimensional paper electrophoresis-paper chromatography technique, which gives a reproducible pattern of spots, detected by the ninhydrin and/or the chlorine test. This technique has enabled them to survey a number of virus strains in comparative fashion (27). Indistinguishable peptide patterns were obtained for strains of very similar amino acid composition (masked vs. common TMV), while differences were observed for strains showing different composition. Most of the peptides obtained from similar digests by the various techniques of isolation discussed above have been cross-identified.

3) The amino acid composition of most peptides has been determined by the Levy FDNB method (28, 29) after desalting the preparations according to Hirs *et al.* (26), or more recently by the method of the Haugaards (30). The N-terminal amino acids were determined by standard procedures (28, 29). The C-terminal amino acid was generally established by the enzyme specificity, but was occasionally ascertained by hydrazinolysis (31) or carboxypeptidase. Sequences have been determined in only a limited number of peptides, ranging from dipeptides to hexapeptides. One hexapeptide isolated from chymotryptic digests as the DNP derivative appeared of particular interest because it proved to represent the C-terminal peptide of the protein (32). The same hexapeptide was obtained from the masked and YA strain, but one of slightly different composition was obtained from the H.R. strain.

4) The identified fragments are devoid of key amino acids and too small to permit even a beginning of the final stage of the work, the fitting together in proper sequence. The disturbing possibility must also be faced that all peptide chains need not necessarily be identical. An appreciable heterogeneity within that population would render our aim, as now approached, unrealizable. At present, all one can definitely say is that all or almost all peptide chains have the same C-terminal sequence. It is also definitely established and agreed (33, 34) that none or almost none of the chains have a free N-terminal group. Several preliminary notes by Braunitzer and Schramm discussing the nature of the 'hidden' N-terminal group and describing a crystalline N-terminal trypsin fragment of the protein have not been confirmed. Some of the claims and ideas were so logically appealing that only with feelings of regret have they been sacrificed, one by one, to the impartial fire of experimental facts.

In conclusion, then, we can state with some assurance that TMV protein is composed of peptide chains of a molecular weight of about 18,000 which lack a free amino end and have a -thr-gly-ser-pro-ala-thr sequence at the carboxyl end. About six such chains form a definite aggregate (A-protein) resembling native proteins in having masked —SH groups and tyrosine-carboxylate bonds. If the —SH groups are hydrogen-bonded, it must be as acceptors, since their H atoms are free. The A-protein tends to aggregate to particles resembling in shape the original virus, but lacking its stability. In the degradation of the virus, about 4000 new groups become titrable between pH 3.5 and 7, which may be masked carboxyl groups, hydrogen bonded in the undissociated form. To these bonds may be attributed the remarkable stability and other surface properties which differentiate the intact virus from the isolated protein.

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AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

Symposium on Chemistry and Metabolism of Phosphatides[1]

Chairman: WARREN M. SPERRY

INTRODUCTION

WARREN M. SPERRY

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IN THIS time assigned to the chairman I shall say a few words in memory of a man who, had he not met a tragic, untimely death, would undoubtedly be a participant in this symposium, probably its chairman. The older members of the audience will have realized that I am speaking of Robert Gordon Sinclair.

I attended my first Federation Meeting in 1924, and Sinclair started a year or two later. As far as I can recall, neither of us missed a meeting up to the time of his death. During those years, through the late 1920's, the 1930's, and even into the early 1940's, the lipide portion of these Federation meetings was a pretty feeble thing, at least in terms of quantity. We rarely mobilized more than a half dozen papers, never enough for a full half-day session, and we usually talked to very small audiences. As Edwin Cohn once expressed it, the lipides were in one of the 'corners' of biochemistry.

We always looked forward to Sinclair's papers; he always had something new and provocative to tell us. He had an inquiring mind. During the early part of his research career, isotopic tracers were not available, but Sinclair devised labeling techniques, rather crude it is true by modern standards, but good enough to provide much new information concerning phosphatide metabolism, some of which has not yet been fully explained. I refer particularly to his demonstration of the rapid uptake of highly unsaturated fatty acids of cod liver oil and elaidic acid by the phosphatides of the intestinal mucosa during fat absorption.

Sinclair was an exceedingly careful, conscientious, and hard-working investigator. Most of his work was done with his own hands. He was full of ideas, for the exploration of which, unfortunately, during the latter part of his career, he had too little time and too few facilities.

I am sometimes disturbed by the absence of references to Sinclair's papers in modern publications. I hope that this is not an indication that his work is being forgotten, but that it has resulted from the pressure of editors on authors to reduce the length of their bibliographies. I urge the younger members of the audience who are working on the phosphatides and did not know Sinclair, to read some of his papers, if you have not already done so. You will find provocative ideas which may be of value to you.

If Sinclair were here, he would be delighted to note that eleven sessions of this Federation meeting are devoted entirely or largely to lipides, but he would not be surprised. He anticipated that there would be the great increase of interest in the lipides and of knowledge concerning them, indicated by the number and high quality of lipide papers being presented during this week. The lipides have certainly come out of the corner. If Sinclair were here, he would join me in congratulating Dr. Stotz and Dr. Carter, who have arranged the excellent symposium we are about to hear. He would be looking forward, as I am, to the many new findings which are going to be presented by the leading investigators in the phosphatide field. I wish more than I can say that he might be here with us.

[1] Chicago, Illinois, April 18, 1957.

SPHINGOLIPIDES[1]

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It is the purpose of this paper to review very briefly the present status of the chemistry of sphingosine (sphingomyelin and cerebroside), to discuss certain aspects of the chemistry of the more complex sphingolipides of brain, spleen and red cell stroma, to present evidence for the presence of a new type of sphingosine-phosphate derivative in egg yolk and finally to outline our recent work on a novel phytosphingosine-containing glycolipide of plant seeds.

SPHINGOSINE, SPHINGOMYELIN, CEREBROSIDE

Four very similar long-chain bases—sphingosine, dihydrosphingosine, phytosphingosine and dehydrophytosphingosine—have been isolated from natural sources. The structures of these substances are shown below. It is proposed to designate the derivative of these bases as sphingolipides.

Sphingosine: $CH_3(CH_2)_{12}CH{=}CH{-}\underset{OH}{CH}{-}\underset{NH_2}{CH}{-}\underset{OH}{CH_2}$

Dihydrosphingosine. $CH_3(CH_2)_{14}\underset{OH}{CH}{-}\underset{NH_2}{CH}{-}\underset{OH}{CH_2}$

Phytosphingosine: $CH_3(CH_2)_{13}\underset{OH}{CH}{-}\underset{OH}{CH}{-}\underset{NH_2}{CH}{-}\underset{OH}{CH_2}$

Dehydrophytosphingosine: $CH_3(CH_2)_x CH{=}CH{-}(CH_2)_{11-x}\underset{OH}{CH}{-}\underset{OH}{CH}{-}\underset{NH_2}{CH}{-}\underset{OH}{CH_2}$

Sphingosine was obtained by Thudichum (1) over 70 years ago from brain lipides, and dihydrosphingosine was isolated from the same source many years later (2). Recent studies on the chemistry of sphingosine have established its structure as D-*erythro*-1,3-dihydroxy-2-amino-4-*trans*-octadecene (for literature review see ref. 3). The stereospecific synthesis of sphingosine by Grob should make this substance more readily available for biochemical studies and is an especially valuable contribution in view of the difficulties encountered in isolating pure sphingosine from natural sources.

The structure of phytosphingosine (except for the stereochemistry of carbons-3,4) has been established by Carter *et al.* (4). Dehydrophytosphingosine (found as yet only in soybean) has been incompletely characterized as an unsaturated derivative of phytosphingosine (5).

On the basis of far from complete studies it appears that sphingosine and dihydrosphingosine are typical of animal tissues and do not occur in plants. Conversely phytosphingosine, which occurs widely in plants (see 6), has not been found in animal tissues. The possible biochemical implications of these observations provide some interesting problems.

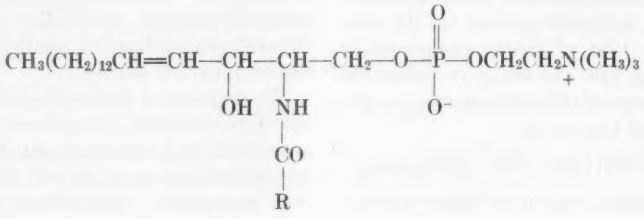
Before taking up the more complex sphingolipides, it seems pertinent to say a word about the occurrence of sphingosine and its simple derivatives. Sphingosine free base has not been isolated from tissues. However, recent studies of blood clotting (7, 8) show that sphingosine is a strong inhibitor of the conversion of prothrombin to thrombin, although at low concentrations it promotes the clotting activity of the lipide activator. Hecht states that sphingosine is a natural plasma inhibitor whose concentration very possibly may be abnormally high in hemophilic

[1] The egg yolk lipide study was supported by a grant from the Procter and Gamble Co. The soybean phosphatide work was done under contract with the U. S. Dept. of Agriculture and authorized by the Research and Marketing Act. The contract was supervised by the Northern Utilization Research Branch of the Agricultural Research Service. The corn phosphatide work was supported in part by a research grant (No. 618-C3) from the Natl. Insts. of Health.

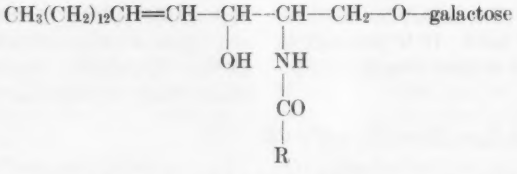
blood (7). The isolation of sphingosinephosphorylcholine from pancreas (9), liver (10) and embryonic tissue (11) has been reported and various N-acyl derivatives of sphingosine (ceramides) have been obtained from spleen (12), liver (13) and red cells (14).

The two best known sphingosine derivatives are the sphingomyelins and the cerebrosides whose structures are shown below:

Sphingomyelin:



Cerebroside:



Earlier workers had established that these substances contain a ceramide residue joined either to phosphorylcholine or to galactose but the point of attachment to sphingosine (i.e., 1- or 3-hydroxyl) was not determined. The cerebroside structure was finally elucidated by Carter and Greenwood (15) by a study of the behavior of hexaacetylphrenosine on catalytic reduction. This substance like triacetylsphingosine yields acetic acid as a hydrogenolysis product thus establishing the attachment of an acetoxyl group on carbon-3 and therefore of the galactosyl group on carbon-1. A similar point of attachment of the phosphorylcholine moiety in sphingomyelin was established by Rouser *et al.* (16) on the basis of oxidation studies in which serine was obtained as one of the final products. This substance could have been produced only if the primary alcohol group of sphingosine had originally been protected by a phosphate ester linkage.

The fatty acid constituents of the sphingomyelins and cerebrosides contain an unusually high proportion of C_{24} acids (lignoceric, cerebronic (α-hydroxylignoceric) and nervonic) and are further characterized by the small amount of mono-unsaturated acids and the virtual absence of polyunsaturated acids. It is interesting to note that cerebronic acid is also an important component of the fatty acids of plant sphingolipides.

GANGLIOSIDE, HEMATOSIDE, GLOBOSIDE

In recent years a number of more complex oligosaccharide derivatives of sphingosine have been discovered containing monosaccharide units other than or in addition to galactose. In a phosphatide symposium it seems inappropriate to discuss these substances in detail but an indication of their chemical nature seems in order. The gangliosides (first discovered in a Tay-Sach's patient (17)) are normal constituents of spleen and nervous tissue (18). They contain both glucose and galactose and in addition N-acetylgalactosamine and the unusual aminosugar acid N-acetylneuraminic acid. Svennerholm (19) has proposed the following structure for the gangliosides:

Ceramide - hexose - hexose - hexosamine - neuraminic acid (N-acetyl).

Strandin, discovered by Folch *et al.* (20) has the same constituents but appears to be of much higher molecular weight (ca. 250,000).

Investigations of the lipides of red cell stroma have brought to light a number of substances, some of which are quite similar to the gangliosides. In 1951 Yamakawa and Suzuki (21) isolated from the stroma of horse erythrocytes a water-soluble sphingolipide which gave the neuraminic acid test. Klenk *et al.* (14, 22) confirmed this finding and reported that human red

cell stroma yielded a different substance, globoside, which contained galactosamine but no neuraminic acid. Further study revealed that dog erythrocytes yield the 'hematoside' type lipide, while those of the sheep, goat and hog contain the 'globoside' type (21, 23, 24). Rabbit and beef erythrocyte sphingolipides contain both hexosamine and neuraminic acid while chicken contains neither (24). Tentative structures proposed for these substances are shown below:

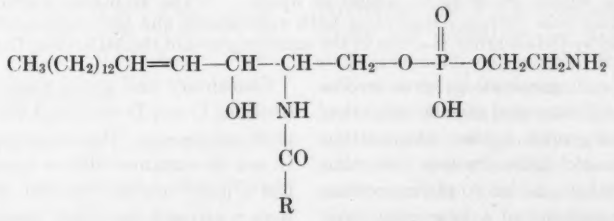
Red cells: Ceramide-$(hexose)_x$-hexosamine
Ceramide-$(hexose)_x$-neuraminic acid

Red cells and brain: Ceramide-$(hexose)_x$-hexosamine - neuraminic acid

It is interesting to note that, whereas the gangliosides contain only galactosamine, the red cell stroma lipides may contain either glucosamine (rabbit) or galactosamine (pig) or a mixture of the two (human, sheep, goat, beef) (25). These complex glycolipides have appreciable water solubility and dissolve with difficulty in organic solvents. Hence, they are difficult to isolate and separate. Much further work remains before the exact nature of these substances is defined.

NEW ETHANOLAMINE-LIPIDE OF EGG YOLK

Although there have been indications in the literature of the possible existence of the ethanolamine analog of sphingomyelin (26, 27) this substance has not been isolated from natural sources.



Recently in a large scale fractionation of egg yolk phosphatides in our laboratories a considerable quantity of sphingolipide was prepared by the mild alkaline hydrolysis procedure of Schmidt *et al.* (28). One hundred gm. of crude phosphatide gave about 1.5 gm of sphingolipide. The crude sphingolipide contained both cerebroside and sphingomyelin as determined by paper chromatography of the intact lipide and of its hydrolysis products. Furthermore, the intact lipide mixture gave a positive ninhydrin test and an acid hydrolysate contained ethanolamine and ethanolamine phosphate. These results strongly indicated the presence of a ceramide-ethanolamine phosphate derivative (tentatively designated as sphingo-ethanolamine). Fractionation of the sphingolipide mixture on silicic acid with chloroform-methanol gave the following results.

Fraction eluted	Vol. % methanol
Ceramide	5
Cerebroside	15
Ethanolamine-lipide + cerebroside	55
Sphingomyelin	70–90

Further fractionation of the mixture of ethanolamine-lipide and cerebroside by column chromatography has not been achieved but solvent fractionation (glacial acetic, pyridine) has given some indication that separation of ethanolamine-lipide in a pure state may be possible.[2] It is interesting to note that the column behavior of the egg yolk sphingolipides is very similar to that of sphingolipides obtained in micro isotope-labeling studies with brain slices (27). It is also interesting to speculate as to the nature of the unusual cerebroside which runs so far behind the other cerebroside components on silicic acid.

INOSITOL LIPIDES OF PLANT SEEDS

PHYTOGLYCOLIPIDE

Following the discovery of inositol as a constituent of soybean phosphatide many investigators have attempted to fractionate this complex mixture and obtain a purified inositol lipide. Lack of satisfactory fractionation methods and of adequate criteria of homogeneity handicapped earlier workers; nevertheless some useful information was accumulated as to the number and type of inositol-containing lipides in corn and soybean phosphatides (29–40). By various solvent fractionation schemes, Woolley (29), Folch (30, 31) and others (32, 33, 40) obtained purified materials of increased inositol content. These prepara-

[2] This compound has now been identified as a long chain ether glycerol-lyso-phosphatide.

tions were reported to contain fatty acids, glycerol, inositol, phosphate and in certain cases one or more carbohydrate components. In the light of what follows it is noteworthy that the only nitrogenous component detected was ethanolamine. Tartaric acid was found by Woolley in a soybean fraction which he called lipositol.

More recently the use of countercurrent distribution techniques by Dutton and co-workers (34, 36) has shown clearly that at least two inositol lipide fractions are present in corn and soybean, one moving with the hexane phase, one remaining in the methanol phase in a hexane-methanol distribution. In the course of these studies galactose, arabinose and mannose were found in various inositol-lipide fractions (34).

In our laboratories a procedure was devised for preparing a purified inositol lipide mixture devoid of glycerophosphatides, sterols and non-lipide contaminants (4). Countercurrent distribution studies on such preparations gave results similar to those of Dutton and gave no indication of affording homogenous lipides. Degradation studies on the inositol lipide fraction from corn were then undertaken and led to the interesting discovery of the presence of a long-chain basic substituent. This substance was shown to be identical with cerebrin base (isolated previously from yeast, moulds, mushrooms (for review see ref. 6) and its structure was established as 1,3,4-trihydroxy-2-amino-octadecane by periodate oxidation studies (4). The base was designated as phytosphingosine because of its origin and close similarity in structure to sphingosine.

Phytosphingosine:

$$CH_3(CH_2)_{13}\underset{OH}{CH}—\underset{OH}{CH}—\underset{NH_2}{CH}—\underset{OH}{CH_2}$$

A mixture of racemic forms of the above structure has been synthesized by Proštenik and Stanačev (41).

With the determination of phytosphingosine providing a further analytical tool a renewed study of countercurrent distribution procedures was undertaken. A detailed investigation of several solvent systems finally provided one (*n*-heptane, *n*-butanol, methanol, water) which gave satisfactory distribution of the components and practically eliminated the usual emulsion problem. Preliminary 30- and 100-transfer distributions gave promising results. In the 100-transfer experiment, three peaks were observed (tubes 6, 24, 56) with considerable overlapping of the first two. In a 400-transfer distribution the slower moving material was further fractionated, although clean separation was not achieved. Analytical data on the various fractions are shown in table 1.

TABLE 1. YIELDS AND ANALYTICAL DATA OF LIPIDE FRACTIONS OBTAINED BY 400-TRANSFER COUNTERCURRENT DISTRIBUTION*

(16 gm lipide were fractionated)

Fraction	Tubes Combined	Recovery, wt.	N Total	N Ref. 42†	P, Total	CHO, Total‡
A	25–45	15.0	0.367	0.111	3.95	none
B	80–95	9.4	0.425	0.165	2.95	none
C	115–140	10.9	1.250	0.531	2.22	8.5
D	150–175	9.4	1.117	0.381	2.91	5.5
E (heptane soluble)	205–235	7.5	0.957	0.190	10.08	4.5
F (heptane soluble)	290–335	3.8	0.303	0.105	2.90	10.8
Crude inositol lipide			0.800	0.310	3.70	6.0

* All analytical values are in % by weight of lipide. † The McKibbin-Taylor procedure (42) measures long-chain base nitrogen (including both sphingosine and phytosphingosine). ‡ Determined as galactose by the anthrone reaction in the aqueous phase of the McKibbin-Taylor hydrolyzate.

Elementary and group analyses showed that fractions C and D contained the majority of the phytosphingosine. The lower partition fractions (A and B) contained little or no carbohydrate and had a lower nitrogen content. From these fractions a nitrogen-free crude phosphatidyl inositol could be obtained by the lead acetate precipitation procedure of Scholfield and Dutton (36).

Although fractions C and D were considerably enriched in long-chain base it was obvious that complete separation would demand a much larger number of transfers, making the preparative application of this procedure extremely laborious. Since the distribution results showed clearly the presence of at least two phytosphingolipides it was decided to subject the original inositol lipide mixture to mild alkaline hydrolysis in the hope of destroying phosphatidyl inositol

and obtaining the phytosphingolipides free of other phosphatides.

Crude corn inositol lipide, when subjected to mild alkaline hydrolysis, yielded an amorphous, insoluble precipitate which was freed of fatty acids by acetone extraction and was further purified by precipitation from pyridine solution with ethanol. The same material was obtained in proportionately lower yield from the original phosphatides. Similar products were obtained from soybean, flax, peanut, wheat and cottonseed phosphatides. The purified phytosphingosine-containing lipides have been designated as phytoglycolipides.

Typical data on the preparation of crude phytoglycolipide are given in table 2, and the properties of purified phytoglycolipide are recorded in table 3.

TABLE 2. PREPARATION OF CRUDE PHYTOGLYCOLIPIDE FROM INOSITOL LIPIDES OR CRUDE PHOSPHATIDES OF PLANT SEEDS

Starting material	Inositol Lipide		Total Phosphatide	
	Corn	Flax	Soybean	Peanut
Analyses, %				
N	0.94	0.92	0.99	3.21
P	4.83	3.46	2.79	1.27
LCB-N*	0.32	0.41	0.056	0.051
Thannhauser-Schmidt product				
Yield, %	37.0	28.0	7.2	11.0
Analyses, %				
N	1.53	1.57	0.62	0.81
P	2.66	2.06	0.49	1.10
LCB-N*	0.75	0.80	0.37	0.40

* Long-chain base nitrogen.

TABLE 3. PURIFIED PHYTOGLYCOLIPIDES

	Corn	Soybean	Flax	Peanut
Analyses				
N	1.88	1.66	1.68	1.73
P	1.93	2.07	1.84	1.93
LCB-N	0.73	0.79	0.83	0.64
$[\alpha]_D$ (pyridine)	+50°	+50–55°	+47°	+50°
Anthrone	20	20	13	14

The purified phytoglycolipide samples were obtained as white, amorphous powders, insoluble in all the common solvents except basic ones such as pyridine, morpholine and aqueous alkali. The material prepared by precipitation from pyridine was ash-free and gave a negative sterol test. In each case the nitrogen phosphorus ratio was about 2:1 and the long-chain base nitrogen constituted about half of the total nitrogen.

The electrophoretic behavior of phytoglycolipide samples from corn and soybean was studied in buffered solutions prepared by dissolving the lipide in aqueous base and dialyzing the solution against buffers of the appropriate pH. Each sample behaved as a mixture of two acidic components; with corn the ratio of the two components was about 65:35; with soybean 95:5. In preliminary ultracentrifugal studies two components were again observed.

The existence of two different sphingolipides has thus been indicated by the countercurrent distribution study and by electrophoretic and ultracentrifugal patterns. It is of interest that the proportion of cerebronic acid in corn and soybean phytoglycolipides corresponds to the weight distribution in the electrophoresis experiments. Whether this correspondence has any significance remains to be determined.

Purified phytoglycolipide gave strong Molisch and anthrone tests, and a positive Scherer inositol test. A weak and somewhat atypical ninhydrin test was obtained. In order to determine the constituent moieties of phytoglycolipide, paper chromatographic studies were made of the acid hydrolysis products. Hydrolysis with 0.5 N sulfuric acid liberated galactose, arabinose and mannose in approximately equivalent amounts. (Each of these sugars had previously been shown to be present in inositol lipide fractions (34, 36). Hydrolysis with 6 N hydrochloric acid gave one strong and several weaker ninhydrin positive spots. The main spot gave an Rf value identical with that of glucosamine, and D-glucosamine hydrochloride was isolated from the hydrolysate and characterized by analyses, infrared spectrum and X-ray powder pattern. No ethanolamine or serine could be detected and it was discovered that D-glucosamine under the hydrolysis conditions employed gave the same pattern of ninhydrin positive spots as did the phytoglycolipide samples. This is, to the best of our knowledge, the first reported occurrence of hexosamine as a constituent of a plant lipide.

Inositol was isolated from acid hydrolysates and identified by physical properties and analyses.

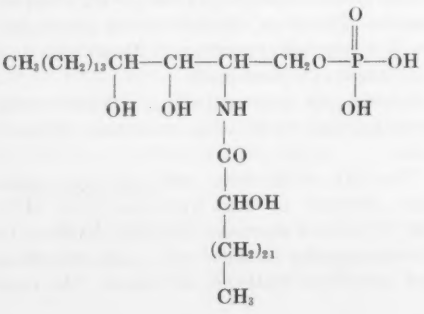
The fatty acids from corn phytoglycolipide were liberated by acid hydrolysis (6 N HCl/100°/6 hr.) and separated into three fractions by chromatography on silicic acid, using chloroform and chloroform-methanol as eluants. The main

fraction (65%) was identified as cerebronic acid by melting point, analysis and infrared spectrum. A further fraction (30%) was identified as lignoceric acid and the remainder consisted of lower melting fatty acids. The fatty acids from soybean phytoglycolipide appeared to be mainly (95%) a mixture of palmitic and stearic acids (50:50) with the remainder consisting of cerebronic acid.

Phytosphingosine was isolated from soybean phytoglycolipide by hydrolysis with saturated barium hydroxide (6 hr. reflux). The material obtained by extracting with chloroform, after acidifying to pH 1, was triturated with 5% potassium hydroxide and extracted with ether. Recrystallization of the extracted material from ether gave a purified base, m.p. 91.5–93°, whose infrared spectrum was identical with that of authentic phytosphingosine except for the presence of a *trans* double bond peak at 970 cm^{-1}.

In order to obtain partial degradation products of phytoglycolipide the barium hydroxide hydrolysate was further investigated. The material obtained by extracting with chloroform was fractionated on silicic acid using chloroform containing varying proportions of methanol as eluant. Impure fractions containing fatty acids, ceramide and ceramide phosphate were obtained. A final elution with 0.3 N methanolic hydrogen chloride gave a fraction which, after recrystallization from methanol-acetic acid, gave a material melting with decomposition at 160–162°. Analytical values were consistent with those of (cerebronyl) ceramide phosphate.

(Cerebronyl) ceramide phosphate:



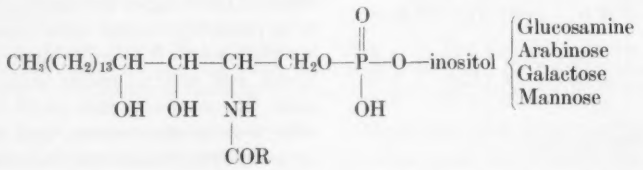
The dipotassium salt was obtained by suspending the material in warm methanol and adding 2.5 moles of potassium hydroxide. On cooling the salt precipitated out. The analytical values were consistent with those of the dipotassium salt of (cerebronyl) ceramide phosphate.

Periodate oxidation of the ceramide phosphate followed by permanganate oxidation and hydrochloric acid hydrolysis (6 N) gave two ninhydrin positive spots running on papergrams identically with serine and serine phosphate. These results indicate that the phosphate group of ceramide phosphate is attached to the primary hydroxyl group of phytosphingosine.

The water soluble material from the barium hydroxide hydrolysis of the phytoglycolipide was treated with Dowex 2 (bicarbonate phase) to remove acidic components and from the eluate an oligosaccharide was isolated which on hydrolysis showed the presence of arabinose, galactose, mannose, inositol and glucosamine.

Elution of the Dowex 2 with dilute acid gave a further phosphorylated oligosaccharide which showed the same carbohydrate components on acid hydrolysis.

The following tentative structure was advanced for phytoglycolipide on the basis of the above chemical evidence plus the fact that inositol monophosphate has previously been isolated from crude inositol phosphatides.



Treatment of phytoglycolipide with 0.3 N methanolic hydrogen chloride gave further information regarding its structure. Most of the lipide dissolved on refluxing and after cooling a white amorphous solid precipitated. Purification of this material by silicic acid chromatography gave an ash-free product devoid of arabinose, galactose and mannose. This product gave a blue color with ninhydrin in pyridine. To our surprise, however, the infrared spectrum showed a strong absorption at 1725–1730 cm^{-1}, indicating the presence of a methyl ester group. Furthermore elementary analyses for this material were lower than the calculated values for a ceramide-phosphoryl-inositol-glucosamine compound, suggesting the presence of a here-to-fore undetected

moiety which on the basis of the infrared spectrum must contain a carboxyl group.

Woolley (29) had reported the isolation of tartaric acid from lipositol but this acid could not be detected in hydrolysates of phytoglycolipide. In order to obtain further information concerning the nature of the acidic component, an investigation was made of the hydrolysis products of the oligosaccharide obtained from the barium hydroxide hydrolysates. The oligosaccharide was purified by several precipitations from aqueous solution with ethanol and was finally obtained as an ash-free white powder.

When the oligosaccharide was treated with concentrated mineral acids only inositol, glucosamine, galactose, arabinose and mannose could be detected in the hydrolysate. No evidence for an additional component was obtained. Under somewhat milder conditions galactose, arabinose and mannose were cleaved off, leaving a ninhydrin-positive non-reducing material which was obtained in pure crystalline form by chromatography over cellulose (isopropanol:acetic acid:water 3:1:1) Analyses of the recrystallized material showed that it contained inositol, glucosamine and a third component corresponding approximately to a C_6 carbohydrate unit. However, drastic acid hydrolysis of this material gave (by paper chromatography) only inositol and glucosamine.

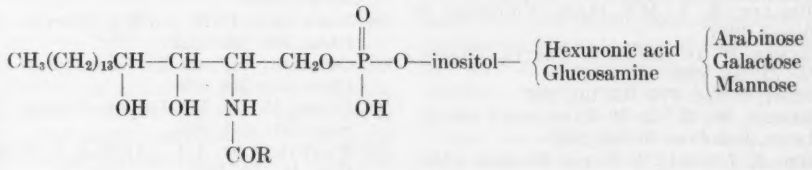
The presence of a carboxyl group as indicated by the methanolysis experiments, the analytical data for the above 'trisaccharide' degradation product and the known instability of hexuronic acids toward strong mineral acids suggested that the "trisaccharide" might contain a hexuronic acid in addition to inositol and glucosamine. This idea was supported by the strong carbazole uronic acid tests (43) given by both the oligosaccharide and the trisaccharide. It was confirmed by the isolation from the partial acid hydrolysis of the trisaccharide of a substance showing properties similar to those reported for hyalobiuronic acid (glucuronido-glucosamine) (44).

The above chemical evidence allows the tentative structure A to be assigned to phytoglycolipide.

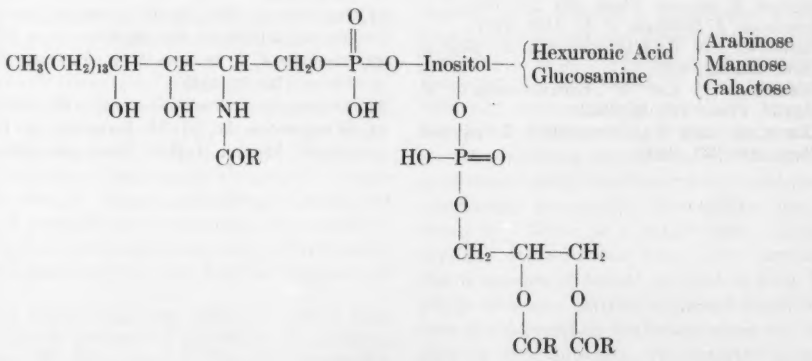
It seems certain that all the components of phytoglycolipide have now been accounted for. However, phytoglycolipide must be considered a generic name since the isolated material consists of a family of lipides differing in fatty acid constituents.

Phytoglycolipide is unique among the sphingolipides in possessing the structural features of a glycolipide as well as those of a phosphatide.

There remains, finally, the question as to the nature of the original phytosphingolipide present in the inositol lipide fraction. The inositol lipide is quite soluble in a variety of organic solvents



Structure A



Structure B

while phytoglycolipide is a much more polar compound and is soluble only in a limited number of basic solvents. It seems obvious that some sort of fatty acid residue is eliminated in the preparation of phytoglycolipide from the inositol lipide mixture. This conceivably might represent fatty acids attached by ester linkages directly to the carbohydrate constituents of phytoglycolipide. However, it is interesting to note that glycerol is present in the phytosphingosine-containing fractions from the 400-transfer countercurrent distribution of corn inositol lipide. This suggests the possible presence of a phosphatidyl moiety attached to phytoglycolipide. The structure B would be consistent with analytical data (N and P) obtained on the countercurrent distribution fractions and would be expected to yield phytoglycolipide on mild alkaline hydrolysis.

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DISCUSSION: GLYCOLIPIDE CHROMATOGRAPHY[1]

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WITH the end in view of studying the metabolism of the brain glycolipides, particularly with isotopes, certain restrictions were imposed in considering the development of methods. First, the methods should be usable on a small (milligram) scale; second, the yields in isolation should be quantitative, or nearly so; and third, phospholipides and other contaminants should be discarded as completely as possible. Evidently chromatographic methods would be necessary.

The first approach tested was the use of ion exchange resins, using resins of high porosity. When a crude extract of brain lipides was passed through a column of Dowex 50-X4, a fair amount of phospholipide was taken up (1). Presumably this is primarily choline-containing material. The lipides tend to leak off the column with further washing so that the separation does not seem to be clean. Strongly basic resins were next tested but it was found that halogen-containing solvents react vigorously with the resins. Solvent systems that do not contain a halogenated solvent are rather poor for glycolipides. Duolite A-7, a weakly basic resin, was found to take up some of the lipide in the effluent from the Dowex 50 column. This fraction includes sulfatide, some phosphatides, and gangliosides. Differential elution of these fractions from such a column has been reported by Svennerholm (2). It was next found that Florisil, an activated magnesium silicate, would adsorb virtually all the phosphatide in brain, as well as the gangliosides. Of the lipide phosphorus, 0.6% passes through the Florisil and presumably this represents a novel type of phospholipide. Half of this phosphorus is absorbed by a mixture of ion exchange resins; perhaps this fraction represents phosphosulfatide. The effluent of a brain lipide extract from a Florisil column contains all the original sulfatide and cerebroside. The former is absorbed when the material is next passed through a mixture of ion exchange resins, and the cerebrosides, together with other nonionic materials, particularly cholesterol, appear in the effluent. These observations formed the basis of methods for determining brain cerebrosides (1) and isolating cerebrosides of high purity on a preparative scale (3). The sulfatide can be eluted from the resins with lithium acetate in chloroform-alcohol-water and is recovered from the effluent by concentrating and then precipitating with barium chloride. Lithium acetate was chosen because of its high solubility in organic solvents. Acid was not used for elution because of the danger of hydrolyzing the sulfate esters.

To purify the cerebrosides further, a partition column with polystyrene beads (unsulfonated Dowex 50) and toluene-alcohol-water was used. A trace amount of a glycolipide came off first, then cerebrosides, and then cholesterol. We were unable to fractionate the individual cerebrosides with this system.

By taking advantage of the high water solubility of gangliosides it was possible to carry out a turnover study in rat brain with radioactive galactose (4). It was found that the gangliosides and cerebrosides exhibit turnover but the sulfatides are not degraded. In order to study the individual cerebrosides, the silica column of Weiss (5) was applied to whole brain lipides. The fractions were analyzed by a modified anthrone method (1) and the glycolipide peaks found by Weiss were observed; band II was by far the largest. Judging by the number of anthrone-positive peaks, the silica column does not separate all the cerebrosides known to occur in brain. Two bands, well defined, gave strong brown colors with the anthrone reagent, so it would appear that anthrone shows a somewhat different reaction with at least two major lipide constituents.

Recently we have been studying the glycolipides of red cells, some of which have been shown to resemble the gangliosides. Yamakawa and coworkers have isolated a glycolipide, globoside, from human stroma and claim that this substance possesses A, B and O blood group substance activity (6). We have extracted whole dry red cells with hot methanol-chloroform and have chromatographed the resultant lipides on a cellulose column, following the effluent with anthrone. A surprisingly complex pattern of anthrone-positive substances, presumably glycolipides, has been found (7). There is a major peak, sometimes partially resolved into two peaks; presumably this represents globoside, at least in part. Blood group substance activity is indeed found in this area as evidenced by inhibition of red cell agglutination with anti-sera. The activity is specific for the A and B groups. In the case of AB red

[1] This research has been aided by a grant from the Multiple Sclerosis Foundation of America, Chicago, Ill. (Dr. Lewis J. Pollock, responsible investigator).

cells, partial separation of the A and B substances occurred. The zone of maximal blood group activity does not appear close to the peak given by anthrone analysis and it would appear that the activity is found in only a very small part of the major carbohydrate peak. Substantially similar results were obtained when the extract was prepared by a method not involving heat. The complexity of the elution curves is intrinsically interesting and it may be that the method will resemble the electrophoretic analysis of plasma proteins in clinical value.

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STRUCTURE OF LECITHINS, INOSITOL PHOSPHATIDES AND ACETAL PHOSPHATIDES

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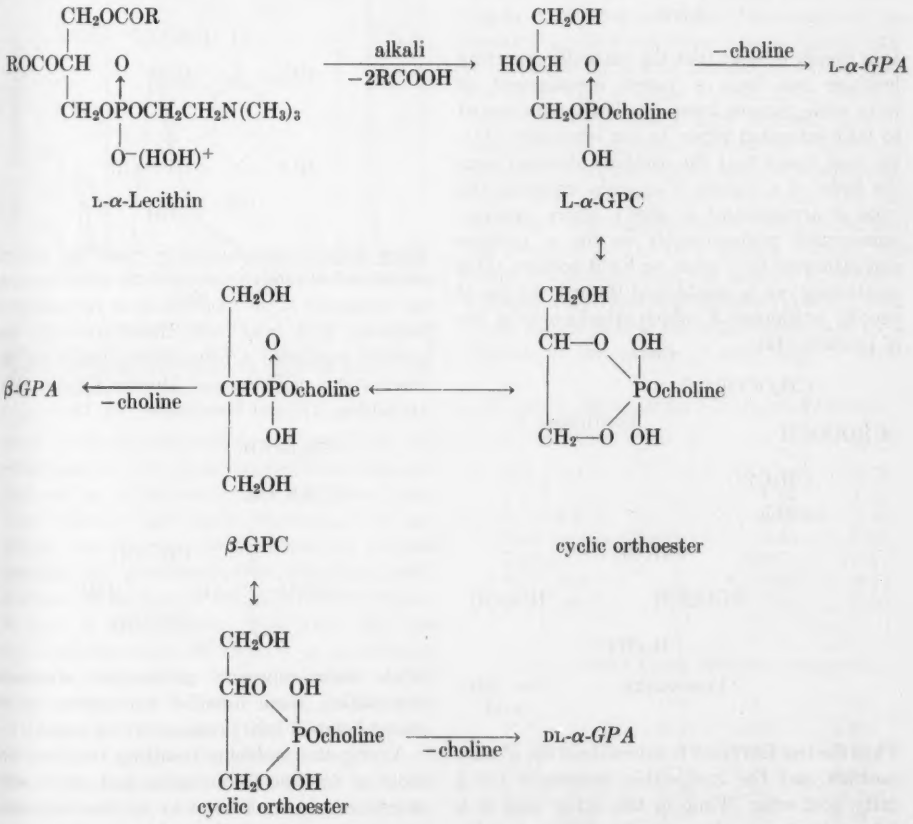
Although the group of phospholipides represented by the lecithins, inositol phosphatides and acetal phospholipides have been well recognized for many years, they still present a formidable biochemical problem. While the basic composition of naturally occurring lecithins has been accepted for many years, it was not until recently that conclusive evidence was presented for their stereochemical configuration. Less definitive evidence is available on the structure of the phosphoinositides and acetal phospholipides. Thus without adequate or complete information on the chemical structure of these lipides, it is not surprising that the lipide biochemist may have relatively little information on the role of these compounds in biochemical processes.

Perhaps the major hindrance to the suitable elaboration of the structure and function of the phospholipides has been associated with their isolation and purification. In addition to oxidative changes, enzymatic alterations and general lability of certain of the bonds in these molecules during isolation, adequate procedures for their purification have only recently been forthcoming. Furthermore, it can be said with a great deal of certainty that the lipide biochemist cannot count crystallinity as one of his colleagues in these efforts and hence may have to be content or ecstatically overjoyed with viscous oils or highly amorphous compounds. However, notwithstanding these facts, considerable progress has been made in our knowledge of these compounds and it is the intent here to dwell on some aspects of the chemistry of these three phospholipides.

LECITHINS

If one were to categorize any of these compounds as a classical phospholipide, lecithin would undoubtedly occupy this position. Inasmuch as it is widely distributed in significant amounts in a variety of plants, microorganisms and animals, this phospholipide has been the subject of many investigations. Due to the classical cadmium chloride procedure of Levene and Rolf (1), lecithin has been one of the easiest of the phospholipides to isolate in a fair state of purity. Although not actually isolated in a state of high purity until rather recently (2, 3), nevertheless, the basic structure of lecithin had been correctly deduced several years ago. However, until the recent excellent studies of Baer and his colleagues (4, 5), the stereochemical configuration of the native lecithins was never satisfactorily explained. Their investigations resulted in the unequivocal synthesis of a series of saturated

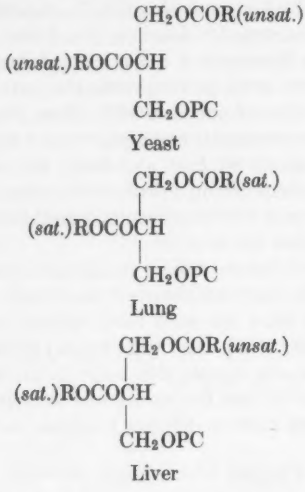
L-α-lecithins, namely, dimyristyl, dipalmityl and distearyl, and more recently, a fully unsaturated type, (di-oleyl)-L-α-lecithin. This tour de force then allowed a strict comparison of the properties of the native lecithins with those of the synthetic lecithins of known structure and configuration. These observations gave strong support to the propositions that only the L-α- form of lecithin existed in nature. However, it was necessary to explain the reports on the occurrence of β-lecithins (6). Pursuant to this point, Baer and Kates (4) showed that the products resulting from the controlled hydrolysis of L-α-glycerylphosphorylcholine (GPC), L-α-lecithins and related compounds were such as to cast doubt on the validity of the α and β isomer proposal. They found that intramolecular migration of the phosphate group had occurred on hydrolysis with the formation of the previously observed α and β forms of glycerophosphate. This can be formulated as follows:



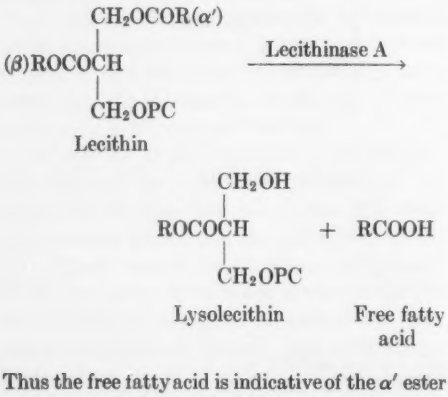
Hence, at present, the findings of β-lecithins must be attributed to an artifact of the hydrolysis procedure. These observations have been confirmed by Long and Maguire (7). In addition, in our laboratory Dr. Uziel (8) found that lysolecithinase B attacks a lysolecithin, derived enzymatically from lecithin, with the formation in good yields of only L-α-GPC. Thus, these combined experimental findings give good support to the proposal by Baer and Kates (4) that only L-α-lecithins are in existence in nature and on the basis of present evidence, β-lecithins must be considered not to occur.

It is of interest from a comparative standpoint that the lecithins obtained from many diverse sources have the same basic skeletal structure, L-α-GPC, with the fatty acids as the only variant. As regards this latter point, it is important to note the nature and arrangement of the fatty acids on different lecithins. An interest-

ing comparison can be made when one examines the lecithins obtained from three different sources, i.e., yeast (9), lung (10) and liver (11):



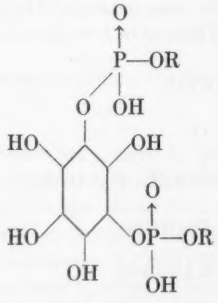
It is clearly evident that the naturally occurring lecithins can have a varied arrangement of fatty acids, ranging from completely unsaturated to fully saturated types. In our laboratory (11), we have found that the lecithins obtained from the livers of a variety of animals contained the type of arrangement as shown above, namely, unsaturated predominantly on the α' position and saturated fatty acids on the β position. This positioning can be established through the use of specific lecithinase A, which attacks only at the α' position (12):



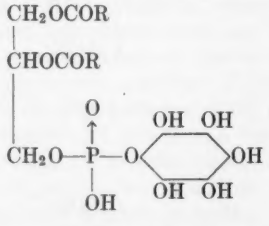
Thus the free fatty acid is indicative of the α' ester position and the lysolecithin represents the β fatty acid ester. While in this latter case it is interesting to consider a possible difference in the metabolic reactivity of the two ester groups, further investigation is necessary to establish the significance of these observations.

PHOSPHOINOSITIDES

Perhaps one of the more undisciplined areas of phospholipide chemistry today centers about the phosphoinositides. Inasmuch as it shall not be possible to review the entire field at this time, reference is made to the recent excellent review on this subject by Folch and LeBaron (13). In brief, although the structure of the phosphoinositides has not been precisely defined, it is apparent that myo-inositol is present in these lipides in the esterified form, either as the mono or diphosphate. Folch (14) in his classical work on the phosphoinositides of brain proposed the following partial structure:



where inositol-m-diphosphate was the central component with glycerol and fatty acids forming the remainder of the molecule in as yet unknown positions. In a brief note, Hawthorne (15) has recently suggested a formulation, based on the observations of Faure and Morelec-Coulon (16), McKibbin (17) and Hawthorne (15, 18):



While these represent provocative structural possibilities, more detailed information is required before a final proposal can be made.

Among the problems besetting this area are those of suitable fractionation and purification procedures. In our laboratory we have been concerned recently with the isolation and structure

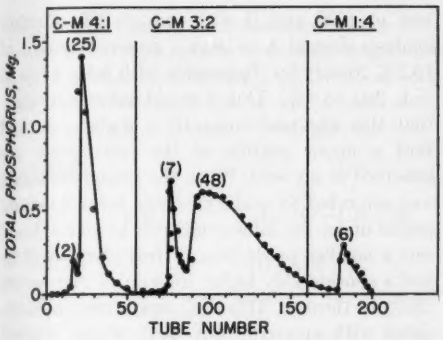


FIG. 1. Chromatography of beef liver mixed phospholipides on silicic acid- hyflo super cel columns. The eluting solvents were chloroform (C)-methanol (M) mixtures (v/v). The first fraction (A) obtained in the C-M 3:2 elution contained the phosphoinositide component.

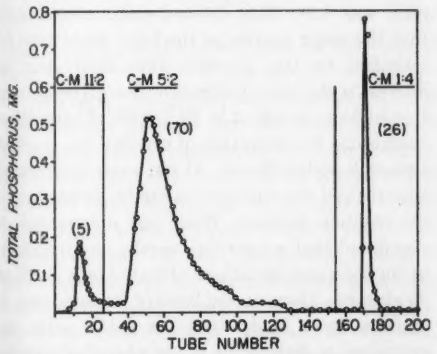


FIG. 2. Rechromatography of C-M 3:2 A fraction (fig. 1) on silicic acid- hyflo super cel columns. A further rechromatography of each of these components showed no further fractionation.

proof of the phosphoinositides of beef liver and other sources (19). These studies have been carried out in collaboration with Dr. June Olley, John Dittmer and Emily Warashina. In particular, our efforts have been directed towards isolation of a nitrogen-free phosphoinositide fraction. On the basis of evidence in the literature to date, it would appear most likely that the major phosphoinositide fraction in mammalian tissues is nitrogen-free. Concomitantly it was, of course, important to learn more of the structure of these complex phospholipides. Experimentally, when the mixed phospholipides of beef liver (A) are chromatographed on silicic acid, with the concomitant use of chloroform-methanol mixtures (4:1, 3:2 and 1:4, v/v) approximately 95% of the phosphoinositides can be obtained in one fraction, namely, the initial component in the C-M 3:2 eluate (fig. 1). This phosphoinositide mixture can then be further fractionated, with excellent recoveries, by rechromatography on silicic acid. Two fractions are obtained, one representing 70% of the total (I) and the other, 20% of the total (II) (fig. 2). An indication that these latter fractions are not artifacts of the chromatography procedure is given by the reproducibility of different runs and also by rechromatography of the individual fractions on silicic acid. In the latter case no conversion of one fraction into the other is apparent. The composition of these two major fractions (I, II) is recorded in table 1. It is evident that both are inositol containing phospholipides, with I having a small contaminant amount of nitrogen and II a significantly higher level of nitrogen. In addition, it was of interest that fraction I contained demonstrable amounts of free glucose, while II contained only trace amounts. This sugar can be removed by the Folch water wash technique (20). A particular point that has emerged from these studies is that a crude phospholipide fraction, which originally has a high pigment content, tends to yield inositide fractions of rather high nitrogen values. In a second series of experiments, a beef liver phospholipide preparation (B) was obtained which was considerably less colored than the previous batch. In a similar manner, the major inositide fraction was isolated by chromatography and rechromatography on silicic acid. These results showed the major component to be present to the extent of 85% with a minor

TABLE 1. BEEF LIVER PHOSPHOINOSITIDE [A]

	I	II
N/P, m.r.	0.19	0.38
FA/P, m.r.	1.92	1.30
=/mM F.A.	0.78	0.49
Free glucose/P, m.r.	0.40	0.04
Unsap.	<3	<0.9
In/P, m.r.	0.90	0.70
gly/P, m.r.	1.01	0.96

TABLE 2. BEEF LIVER PHOSPHOINOSITIDE B

	Orig.	I	II
P, %	3.53	3.40	3.80
N, %	0.23	0.095	0.98
N/P, m.r.	0.14	0.06	0.57
FA/P, m.r.	1.92	1.90	Not run
In/P, m.r.	0.86	1.19	0.67
gly/P, m.r.	1.00	1.00	1.30

inositide fraction representing 7% of the total, and this has proved the most repeatable distribution of these components. An additional 5% of a fast-moving, highly-colored inositol free component was isolated. Thus, 97% of the phosphorus was recoverable. The analysis of these two phosphoinositides is shown in table 2.

As indicated by the data in table 2, the major phosphoinositide fraction (I) is nearly nitrogen-free while the minor inositide component (II) has a relatively high nitrogen content. Most attention has been directed towards the chemical characterization of fraction I. This component can be obtained as a very light tan powder, not easily oxidized by exposure to the atmosphere. While various preparations have shown a free glucose content ranging from 2 to 8%, this contaminant sugar can be removed by washing by the Folch technique (20). It is interesting to note that the major amount of any free sugar present in the original phospholipide mixture chromatographed on silicic acid tends to localize in the inositide fraction. The analytical data on the composition of I showed it to contain myo-inositol, glycerol, fatty acids, phosphorus and a residue (metal). This accounted for approximately 95% of the molecule, which can be classified as a monophosphoinositide.

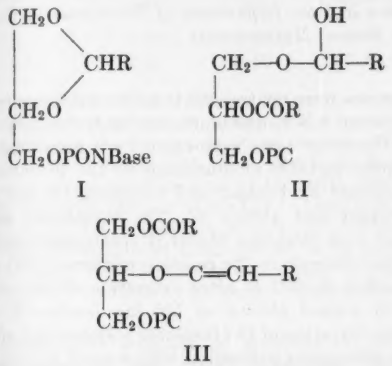
In various reports on the structure of phosphoinositides, the location of the fatty acids has never been satisfactorily explained. Although it has been provocative to consider that they are esterified to the glycerol, no conclusive evidence has been forthcoming. A short term hydrolysis of this phosphoinositide provided some very interesting results. Consequently, our initial efforts have been to ascertain, if possible, the sites of esterification. When the phosphoinositide (I) was treated with 2 N HCl at 98° for 30 min., free glycerol, fatty acids, a diglyceride, inositol monophosphate and free inositol were obtained. No inorganic phosphate is released in this period. In agreement with the observations of Folch (14) for the brain diphosphoinositide and Hawthorne for the soybean inositides (18), it was found that all the phosphate became water soluble and was still organic in nature.

An examination of the 'fatty acid' fraction (P free) revealed it to have a high neutral equivalent (590), indicating the possible presence of glyceride and free fatty acid. When this fraction was treated with sodium carbonate, it was separated into two components, A, with a neutral equivalent of 1200 and B with one of 260. Further analysis showed A to have a glycerol content of 15.3% (theory for diglyceride with fatty acids of N.E. 260, 15.0%). Thus it would indicate strongly that this was predominantly a diglyceride and that a major portion of the fatty acids are esterified to glycerol. When the phosphoinositide was subjected to acid hydrolysis for a 2-minute period under the same conditions as above, there was a smaller production of free glycerol (9%) and a considerably higher amount of diglyceride (50% of theory). This diglyceride was contaminated with approximately 30% of the original phosphorus-containing compound, but this could be removed by countercurrent extraction with petroleum ether-85% ethanol. This glyceride fraction (P free) had a neutral equivalent of 3267 and a glycerol content of 14.8% (theory for diglyceride, 15.0%). The fatty acid to glycerol ratio was 1.94. This showed quite conclusively that the major portion of the fatty acids must be esterified to the glycerol. One final item of interest is the rate of release of free glycerol from I on hydrolysis with 2 N HCl at 98°. Under these conditions, the liberation of glycerol has reached a plateau within 60 min. At the latter time period a good yield of a diglyceride can be isolated from the reaction mixture. While not unexpected, it was found that a pure diglyceride, dipalmitolein, under the same conditions liberated only 0.1% of its glycerol. Thus, on the basis of this evidence, it appears most likely that the fatty acids are presented as diglyceride in the phosphoinositides of beef liver and that a phosphate bridge links this diglyceride to an inositol molecule. The rather high lability of certain bonds of this compound emphasizes the need for caution in preparation of derivatives of the intact molecule.

ACETAL PHOSPHATIDES

Finally, attention shall be directed towards recent evidence presented for the structure of the acetal phospholipides. These phospholipides are present in relatively high amounts in brain, heart muscle and sperm, while they are found to a very limited extent in liver, plasma and intestinal phospholipides. At the present time two proposals, differing from the classical concept (I), have been made for the structure of the naturally occurring acetal phospholipides. Klenk and Debuch (21) claim that the choline containing acetal phospholipides of beef heart muscle are best represented by the hemi-acetal structure

(II), on the basis of hydrogenation of the compound to an ether, gentle hydrolysis to give the batyl and chimyl ether of L-α-glycerophosphoric acid, and also conversion of the original compound to lysolecithin and a free aldehyde.



I II

III

Rapport (22), on the other hand, believes on the basis of the stability of the acetals in absolute alcohol, ease of reduction and sensitivity to acid and mercuric chloride, that the structure would be more correctly represented by a vinyl ether-type structure (III). In addition, Rapport (22) observed that lecithinase A of snake venom removed the major portion of the fatty acid of the molecule. Assuming that lecithinase A attacks this compound in the same manner as it does lecithin, the fatty acids must have been primarily on the α′ position. However, as suggested by Rapport, the original plasmalogen may well be present as a mixture of compounds of both α′ and β linked aldehydrogenic chains, with the β type predominating. Further evidence on this structure should prove most informative.

In conclusion, the chemistry of these three varied phospholipides is being investigated with considerable interest in several different laboratories and it appears certain that within the reasonable future their complete structure will be known. This should aid materially in the interpretation of their role in cellular processes.

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DISCUSSION: QUANTITATIVE PARTITION OF ACETAL PHOSPHOLIPIDES AND OF FREE LIPIDE ALDEHYDES[1]

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ONE of the most characteristic properties of the acetal phospholipides is the lability of the bond between the aldehydogenic group and between the glycerol-containing moiety of the molecule toward acids. Hydrolysis of this linkage occurs with Schiff's reagent at room temperature (1) and in tests with phenylhydrazine derivatives under the conditions required for the condensation reactions (2). For this reason, no method for the quantitative differentiation of free lipide aldehydes and of acetal phospholipides is available at present. Even though the development of color in the Schiff test occurs with free lipide aldehydes somewhat more rapidly than it does with acetal phospholipides, Thiele (3) has shown that the analytical usefulness of these differences is rather limited.

We found that free lipide aldehydes could be converted to Schiff-negative compounds under conditions which did not affect the intensity of the Schiff test with acetal phospholipides. It will be shown in this paper that this behavior is a suitable basis for the quantitative partition of lipide aldehydes and acetal phospholipides.

MATERIALS AND METHODS

The behavior of free lipide aldehydes was studied on samples obtained by acid hydrolysis of their dimethyl acetals (4) as well as on acid hydrolysates of emulsions of the total lipides of beef brain and beef heart. Owing to their much greater stability, the dimethyl acetals were hydrolyzed at 100° for 5 min. with 0.2 N hydrochloric acid, whereas the tissue lipides were shaken at room temperature for 3 hr. with 0.2 N hydrochloric acid. All hydrolysates were freshly prepared for each experiment owing to the fact that free lipide aldehydes cannot be stored even for short periods.

Since genuine acetal phospholipides cannot be prepared in pure form, lipide emulsions from beef brain and beef heart were used in the experiments, either as such or after saponification with N sodium hydroxide for 5–16 hr. The saponified samples were neutralized to pH 8 by dropwise addition of 2 N hydrochloric acid under cooling.

The Schiff tests were carried out according to several suitable modifications of the procedures described by Feulgen and Grünberg (5) and by Rapport and Alonzo (6). The extinctions were read in a Beckman Model B spectrophotometer either directly in the reaction mixture at 585 mμ (*method A*, (6)) or after extraction of the color with n-amyl alcohol at 555 mμ (*method B*) or after filtration of the insoluble pigment and after its subsequent extraction with n-amyl alcohol at 555 mμ (*method C*, (5)).

For the determination of water-soluble phosphorus compounds in lipide emulsions, the separation of both phases was achieved by addition of solid ammonium sulfate to 0.8 saturation. This technique yields water-clear aqueous filtrates and prevents hydrolysis of acetal phospholipides.

RESULTS

Behavior of Free Lipide Aldehydes During Incubation of Their Aqueous Emulsions at Different pH Values. Figure 1 shows the relative extinctions obtained in the Schiff test with equal aliquots of a given acid-hydrolsate of a brain lipide emulsion after incubation with buffer mixtures of various pH values for 3 and 16 hr. at room temperature. In the acid range, up to pH 6, the extinction values were not at all or only slightly diminished after the incubation, but in the range between pH 7 and 9, only small fractions of the initial extinction values were observed. The shape of the pH curve of the decrease of the extinctions in the Schiff test was not essentially influenced by the nature of the anions of the buffer mixtures. In the following, the conversion of lipide aldehydes to Schiff-negative compounds during incubation in slightly alkaline emulsions will be referred to as 'aldehyde disappearance reaction' (ADR).

The curves of figure 2 demonstrate that the ADR closely followed first order kinetics until the extinctions had decreased to approximately 10% of the corresponding initial values. The rate of the ADR was not appreciably influenced when the incubation was carried out in an evacuated Thunberg tube. The ADR was completely inhibited by the presence of mercuric chloride at 0.005 M concentration.

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[2] German Exchange Fellow 1955/56.

TABLE 1. PARTITION OF MIXTURE OF FREE LIPIDE ALDEHYDES (ACID-HYDROLYZED BRAIN LIPIDES) AND ACETAL PHOSPHOLIPIDES (NON-HYDROLYZED BRAIN LIPIDES)

Hr of Incubation*	Extinction at 555 mμ (Schiff Test)		
	A†	B‡	Mixture of (A)† and (B)‡
0	0.52	0.58	1.10
16	0.55	0.02	0.52

Stock emulsion of lipides of 20 gm moist beef brain in 100 ml of water. A, 10 ml stock emulsion diluted with 15 ml water. B, 10 ml stock emulsion diluted with 15 ml 0.5N HCl. Aldehyde disappearance reaction with 3 ml of (A), with 3 ml of (B), and with mixture of 3 ml of (A) and 3 ml of (B) at pH 8.3 in presence of 20 ml 0.1M dibasic sodium phosphate in total volume of 30 ml for 16 hr. at 25°. Schiff test (*method C*) in 5-ml aliquots.
*pH 8.3. †Not hydrolyzed. ‡Hydrolyzed.

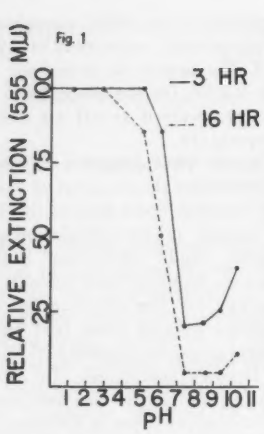
Quantitative Partition of Mixtures of Acetal Phospholipides and Lipide Aldehydes (Acid-Hydrolyzed Acetal Phospholipides). Table 1 shows the results of a representative experiment in which the ADR was carried out under comparable conditions with *a*) an emulsion of brain lipides, *b*) an emulsion of acid-hydrolyzed brain lipides, and *c*) a mixture of both emulsions. The extinction of the mixture which initially equaled the sum of the initial values of (*a*) and (*b*) decreased during the ADR to the value of the initial extinction of (*a*).

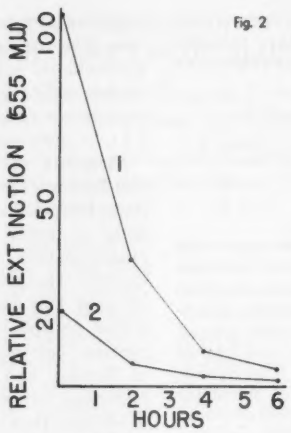
Lipide aldehydes obtained from their dimethyl acetals were likewise converted quantitatively to Schiff-negative compounds under the conditions of the ADR. In these experiments, the lipide aldehydes prepared from their dimethyl acetals were mixed with tissue lipides since pure lipide aldehydes do not form emulsions with water. Lauryl sulfate, which solubilizes lipide aldehydes, could not be used in the ADR since we found that it converts partially the Schiff-negative products of the ADR to Schiff-positive compounds. Furthermore, lauryl sulfate itself in 0.002 M concentration forms a red color of appreciable intensity with Schiff's reagent. The observation concerning the effect of lauryl sulfate will be described elsewhere in detail.

Kinetics of Acid Hydrolysis of Acetal Phospholipides. The availability of a procedure for the quantitative partition of acetal phospholipides and free lipide aldehydes offered the possibility to study the kinetics of the hydrolysis of these compounds by acids. The influence of pH on the course of hydrolysis of these compounds at room temperature is demonstrated in figure 3. At the indicated time intervals, samples of the various hydrolysates were incubated under the conditions of the ADR. The extinctions obtained in the subsequent Schiff tests served as measure for the unhydrolyzed fraction of the acetal phospholipides. The curves show that in presence of 0.2 N hydrochloric acid, the degree of hydrolysis exceeded 95% after 2.5 hr. On the other hand no measurable cleavage occurred at pH 4.8 during 5 hr. at room temperature.

Structure of Acetal Phospholipides. Feulgen and Bersin (7) proposed for the structure of acetal phospholipides of striated horse muscle the formula of cyclic acetals of glycerylphosphorylethanolamines with lipide aldehydes. Subsequently, Thannhauser, Boncoddo and Schmidt (8) adopted this concept for the structure of the acetal phospholipides isolated from beef brain because their general properties agreed with those of Feulgen and Bersin's compounds. In recent historical reviews (9), no mention has been made of the fact that the original paper of Feulgen and Bersin contains a discussion in which these authors emphasized the tentative nature of their interpretations, and in which they suggested explicitly other structural possibilities such as the possibility that their crystalline compounds might be degradation products of the genuine acetal phospholipides, and the possibility of the occurrence of choline-containing acetal phospholipides in tissues.

In 1944, Anchel and Waelsch (10) expressed the view that the lability of the aldehydogenic group of acetal phospholipides was so much greater than that of synthetic glyceryl acetals of lipide aldehydes that an analogous structure of both types of compounds appeared improbable. The first direct evidence for the incompatibility of the cyclic acetal structure of genuine acetal phospholipides with their experimental properties was obtained in 1951 in this laboratory when it was found that no water-soluble phosphorus-compounds were formed during Schiff tests carried out on emulsions of unsaponified brain lipides (11). These observations were recently extended to hydrolysates of brain-lipides with hydrochloric acid since only the results reported in the present paper made it possible to correlate the amounts of water-soluble phosphorus compounds with the degree of hydrolysis of the aldehydogenic groups. Shaking of aqueous emulsions of unsaponified brain lipides with 0.2 N hydrochloric acid for 30′ did not result in measurable increases of the amounts of water-soluble phosphorus compounds over those present in the initial lipide-free filtrates which were obtained by adjusting the pH of the emulsions to 4.5 after the addition of 2 ml of a 1% solution of sodium stearate to 10 ml of the emulsions. The aldehyde disappearance reaction which was carried out on aliquots of the same hydrolysate indicated that 72% of the aldehydogenic groups of the emulsion had been hydrolyzed under these conditions.





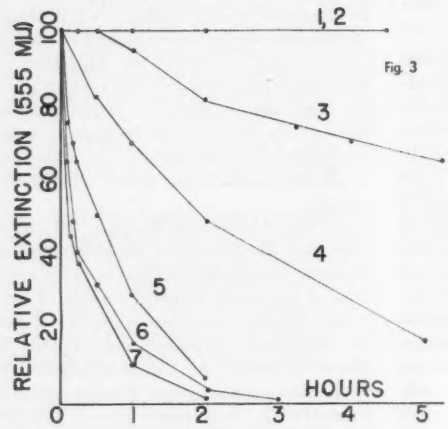


FIG. 1. Influence of pH on rate of aldehyde disappearance reaction of acid-hydrolyzed brain lipides. Hydrolysis in 0.2 N HCl, 24°, 90′; hydrolysate neutralized to pH 7.5 with N sodium hydroxide; 3-ml aliquots containing the hydrolyzed lipides of 0.400 gm moist beefbrain mixed with 20 ml of 0.1 M buffer solutions (sodium acetate buffer for pH 5.1, sodium phosphate buffers for all other pH values). The pH was measured electrometrically in parallel samples. Schiff tests in 3-ml aliquots after 3 hr. (*full line*) and 16 hr. (*dotted line*) incubation at 24°. Total volume of Schiff test, 18.5 ml (*method C*).

FIG. 2. Time course of aldehyde disappearance reaction (ADR) of acid-hydrolyzed brain lipides. *Curves 1* and *2*: lipides of 0.8 and of o,16 gm of moist beef brain respectively. Relative extinctions of both curves expressed as percentages of extinction obtained in initial Schiff test of *curve 1*. Acid hydrolysis, 0.2 N HCl, 24°, 4 hr. ADR, 5 ml, and 1 ml respectively of hydrolysates in total volume of 26 ml. containing 20 ml dibasic sodium phosphate; pH adjusted to 8.5. Schiff tests: Respective aliquots of 1 and 3 ml of ADR samples in total volume of 18.5 ml (*method C*). Initial extinction of *curve 1:* 0.42.

FIG. 3. Influence of pH on rate of hydrolysis of aldehydogenic groups of acetal phospholipides. *Curve 1*, pH 8.5 (sodium phosphate buffer). *Curve 2*, pH 4.8 (sodium citrate buffer). *Curve 3*, pH 3.8 (sodium citrate buffer). *Curve 4*, pH 2.4 (glycine-hydrochloric acid buffer). *Curves 5, 6, and 7*, pH 1.1, 0.9 and 0.78 respectively (0.1, 0.2, and 0.5 N hydrochloric acid respectively). Final concentration of buffer mixtures, 0.1 M. All pH values determined electrometrically on hydrolysate mixtures. Each curve was obtained with a suspension of the lipides of 4.0 gm of moist beef brain in 50 ml at the indicated pH values. At the indicated time intervals, 3-ml aliquots of the hydrolysates were incubated under the conditions of the ADR at pH 8.3 for 16 hr. (total volume 16.6 ml containing 10 ml of 0.1 M dibasic phosphate). The respective extinctions in the Schiff test were interpreted as a measure of the unhydrolyzed groups at the respective phases of acid hydrolysis. Initial extinction of 3-ml aliquot, 0.52 (*method C*).

When the hydrolysis with 0.2 N hydrochloric acid was carried out for 2 hr. with saponified emulsions of brain-lipides (1 N sodium hydroxide, 16 hr., 24°) approximately 50% of the phosphorus of the non-saponifiable phospholipides were converted to water-soluble phosphorus compounds. Since the only phospholipides present as such in saponified emulsions of brain lipides are acetal phospholipides and sphingomyelin, and since the incubation of pure sphingomyelin with 0.2 N hydrochloric acid did not result in any formation of water-soluble phosphorus compounds, it was concluded that the increase of this phosphorus fraction during the acid hydrolysis of saponified lipide emulsion originated from the cleavage of the aldehydogenic bonds of the acetal phospholipides.

These observations show that Feulgen and Bersin's reservations regarding the identification of the crystalline acetal phospholipides with the genuine plasmalogens were justified. They confirm the view first expressed in 1951 by Schmidt (11), that the former differ from the latter by the absence

of a lipide group, owing to its hydrolysis by the alkali treatment which was used in all available procedures for the isolation of crystallized acetal phospholipides. Since then, strong evidence in favor of the fatty acid nature of this lipide group has been contributed from the laboratories of E. Klenk (12) and of M. Rapport (13). Furthermore, Rapport *et al.* demonstrated that the bond between the aldehydogenic group and the glycerol group is not that of a true hemiacetal, but that of a dehydrated hemiacetal, probably that of an α,β-unsaturated ether.

SUMMARY

Lipide aldehydes are practically completely converted to Schiff-negative compounds by incubation of their aqueous emulsions at pH 8.3 for 16 hr. at room temperature. The extinction values of the Schiff test of acetal phospholipides or of their degradation products formed during mild saponification were not appreciably altered under these conditions.

A procedure for the quantitative partition of acetal phospholipides and lipide aldehydes has been described. The conditions of acid hydrolysis of acetal phospholipides have been studied with quantitative methods. Cleavage of 72% of the total amounts of aldehydogenic groups of genuine acetal phospholipides was observed without formation of water-soluble phosphorus compounds. The bearing of this observation on the structure of acetal phospholipides has been discussed.

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NITROGENOUS CONSTITUENTS OF ANIMAL TISSUE LIPIDES[1]

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The complete analysis and resolution of whole tissue lipide extracts is an important and unsolved problem of phosphatide chemistry. An initial part of the solution of this problem is to define and accurately measure the hydrolysis products of the tissue phosphatides. In order to do this, the non-lipide contaminants must be quantitatively removed from the tissue lipide extracts. Several effective procedures hve been devised for this purification (1–4) We have used emulsification with aqueous 0.25 M $MgCl_2$ (1) for purification prior to analysis of the lipide bases, sugars (5), and inositol (6) and have found it satisfactory. It is the purpose of this paper to present some analytical values for the nitrogenous components of animal tissue lipide extracts.

The frozen tissue powders were extracted with Bloor's reagent followed by chloroform (1) and these extracts were then purified of non-lipide contaminants. Total N was determined on the purified extracts by the AOAC Kjeldahl procedure with selenium. Choline was determined as the reineckate salt from a $Ba(OH)_2$ and HCl hydrolysate (1). Sphingosine was determined as chloroform soluble N on this same hydrolysate (7). Ethanolamine and serine were determined from hydrolysates prepared by treating the lipides with 4 N aqueous HCl in a sealed tube at 120°C for 4 hr. (8). The bases were separated on

[1] This work was supported by a grant from the Life Insurance Medical Research Fund.

a permutit column after Artom (9), and color-developed with the 1,2-naphthoquinone-4-sulfonate reagent used by Frame, Russell and Wilhelmi (10, 11). The recovery of serine was quantitative from both synthetic lipide hydrolysates and hydrolysates of whole lipide extracts. Recovery of ethanolamine was 91–100% from most tissues, but only 86–90% from brain. Very little ammonia was formed in the hydrolysis and this did not interfere with the determination. We have found that the values for ethanolamine and serine obtained by this procedure agree well with those obtained manometrically using nitrous acid and ninhydrine (12) respectively. They also agree with those obtained by Axelrod *et al.* (13) from human blood plasma lipides, and by Robins *et al.* (14) from rabbit brain lipides. In both the latter studies the dinitrophenyl derivatives of ethanolamine and serine were measured. However, ethanolamine values are much lower than those obtained by Artom (9) using the periodate procedure on less pure extracts.

Table 1 gives representative values for the component bases of animal tissue lipide extracts. It should be emphasized that the values for the individual bases may include unrecognized substances which determine in the same manner (15). Moreover, the ethanolamine value would probably include hexosamine (16) and neuraminic acid would not be determined at all (17).

The difference between total Kjeldahl N and the sum of the known base N is given as 'undetermined N.' These values are subject to high error since small errors in measurement of the major components will be magnified in the measurement of a minor component by difference. It is probable that direct measurement of the bases will give slightly low values owing principally to artifacts of hydrolysis. Accordingly, the values for undetermined N will be high but the error should not exceed 6% of total lipide N. It appears that brain, lung and blood plasma total N is virtually accounted for by the known bases, whereas intestine, kidney, liver and heart contain substantial amounts of undetermined N. Liver analyses are available from several species and consistently demonstrate 10–17% of undetermined nitrogen. In rat liver this fraction is concentrated in the nuclei and mitochondria (18).

TABLE 1. LIPIDE BASES IN ANIMAL TISSUES

Tissue	Serine	Ethanolamine	Choline	Sphingosine	Undetermined
Dog					
Intestine	14.4	13.8	41.9	17.3	12.6
Brain	15.5	18.5	30.5	30.6	4.9
Lung	12.0	12.0	49.4	21.3	5.3
Kidney	11.6	17.4	40.5	17.9	12.6
Liver	12.5	14.9	46.5	9.1	17.0
Heart	6.0	10.4	45.4	16.6	21.6
Beef heart	11.8	23.0	42.7	14.7	7.8
Human plasma	3.8	3.8	72.0	18.2	2.2
Rat					
Liver nuclei	9.	20.	47.	9.	15.
Liver mitochondria	11.	21.	41.	9.	18.
Liver microsomes	9.	18.	59.	9.	5.
Liver supernatant	12.	19.	50.	16.	3.

Values in % total lipid N (8, 18).

The choline values given do not include carnitine since the latter does not precipitate with reineckate at pH 4–6. Our results demonstrate that no appreciable amounts of carnitine are present in blood plasma lipides, and are in variance with those of Binon and Deltour (19).

The undetermined N in dog (20) and horse liver is concentrated in certain fractions of the alcohol insoluble phosphatides which are under study in this laboratory. We were interested in determining whether this N is *a*) persisting non-lipide impurity N, *b*) an artifact of hydrolysis, or *c*) a hitherto undescribed lipide base. We have therefore initiated some concentration work on the undetermined N of purified dog and horse liver phosphatides. These lipides were hydrolyzed *a*) by refluxing for 16 hr. with aqueous 4 N HCl on a sand bath, or *b*) by 48-hr. room temperature saponification with 1 N NaOH followed by HCl hydrolysis of the chloroform soluble fraction remaining. Both hydrolysates were treated with nitrous acid to remove primary and secondary amines and then precipitated with phosphotungstic acid. This precipitate was regenerated with $Ba(OH)_2$ and the amines chromatographed on Dowex 50. An acid fraction was eluted at pH 7.0 and a basic fraction with 0.1 M Na_2CO_3. Both fractions were high (60–90%) in undetermined N and little more was released by repeated hydrolysis. The combined over-all yields were 3–6% of total original liver lipide N. Work on the identification of these fractions is in progress.

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QUANTITATIVE CHROMATOGRAPHY OF PHOSPHATIDES[1]

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The contributions of chromatography toward the elucidation and simplification of biochemical problems are exceedingly numerous. Yet the application of chromatography to the study of the phosphatides has been quite meager, especially in comparison to similar investigations on other important biochemical compounds. In part, the reason for this procrastination has been the scarcity of pure phosphatides for study, the lability of the native phosphatides, and the difficulty in purifying these compounds because of their non-crystalline nature in the native state. It is the aim of this paper to give results of investigations on both the paper and column chromatography of complex mixtures of tissue phosphatides. In particular, emphasis will be placed on the quantitative aspects of this versatile technique.

The major work on the paper chromatography of the phosphatides on non-impregnated filter paper occurred in the period 1952 to 1956 and was contributed by several laboratories both in this country and abroad (1–10). The solvent systems which were developed were only partially successful and were mainly applicable to rather simple mixtures. Studies from our laboratory (8) on the factors which influence the chromatographic mobility of the phosphatides made it apparent that paper impregnation would be one solution to this problem. The firm binding of the phosphatides to the paper and the limited amount of material which could be used for chromatography on non-impregnated paper presented a formidable barrier which resisted penetration. This problem was circumvented and partially resolved by our finding (7) that acylation of the amino-containing phosphatides prior to chromatography allowed the use of more material and effected a complete separation of phosphatidyl cholines from the cephalins.[3] However, in comparison to the fine paper chromatographic resolution of sugars, amino acids, and steroids, the separation of the phosphatides on non-impreg-

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[2] This work was carried out during the tenure of a Lederle Medical Faculty Award.

[3] The term cephalin includes phosphatidyl ethanolamine, phosphatidyl serine, and the corresponding acetal phosphatides.

nated paper left much to be desired. When alteration of the filter paper itself proved unfruitful (8), paper impregnation seemed more than ever the most likely solution. We therefore investigated the impregnation of the filter paper with mineral oil, rubber, albumin, starch, silicones, various non-volatile alcohols and amides, boric acid and silicic acid. Of these agents, silicic acid was by far the most effective. This finding was not unexpected, since in 1944, Miss Rathmann from our laboratory had found as part of a Ph.D. thesis (11) that silicic acid excelled other adsorbents which she tested in regard to the column chromatography of the phosphatides. Although others (12, 13) have used silicic acid for the separation of the phosphatides from the non-phosphatides, the work of Miss Rathmann appears to represent the first attempt to fractionate the phosphatides themselves by column chromatography on silicic acid.

At the time our experiments were in progress, Lea, Rhodes and Stoll (14) in England published a paper on the chromatographic separation of model mixtures of phosphatides on silicic acid impregnated paper by use of chloroform-methanol as solvent. We then reported (15, 16) the effective separation of complex mixtures of tissue phosphatides on silicic acid impregnated paper by use of solvents containing diisobutyl ketone-acetic acid-water or butyl ether-chloroform-acetic acid-water. Recently Dieckert and Reiser (17) and Brown and collaborators (18) have also obtained useful separations of model mixtures of phosphatides on silicic acid impregnated glass fiber. It is fortunate that there exists at the present time several different solvent systems for the chromatographic separation of the phosphatides. Undoubtedly each system possesses certain advantages over the other. In our hands the diisobutyl ketone-acetic acid-water system has given the best overall separation of a complex mixture of tissue phosphatides and has worked well for two-dimensional chromatography (15, 16). A satisfactory migration of inositol phosphatide and phosphatidyl serine is best attained by water-containing solvents (15).

In order to obtain reproducible chromatograms and the maximum resolution of the phosphatides, the conditions of chromatography must be carefully controlled and kept constant. Some of the variables which were found to affect the mobility of the phosphatides on paper were as follows: the amount of phosphatides applied to the paper and the volume in which they are applied; the size and manner of lining the chromatographic chambers; humidity (especially in regard to the atmosphere that impregnated papers are exposed to during storage); temperature; degree and uniformity of silicic acid impregnation; exposure of impregnated papers to volatile solvents, in particular ammonia, pyridine, and other basic solvents; lipide peroxidation (which causes streaking and very poor resolution); and the manner in which the lipides are extracted from the tissues. The cephalins, such as phosphatidyl ethanolamine and phosphatidyl serine, were found to undergo peroxidation very rapidly even when stored in solvents in the cold. This decomposition was accelerated in solvents containing chloroform and by the presence of hemin. Because of the variability in the mobility of the phosphatides, Rf values alone are not adequate for identification of unknowns; rather it is necessary to compare the mobility of the unknowns with those of authentic samples which are run on the same chromatogram and to rely on specific spot tests.

The success of our investigations was dependent in large measure on the availability of a general detection test for the phosphatides which was simple to carry out, which had the requisite sensitivity, and which would allow for quantitative studies. Rhodamine 6G[4] (colour index 752) proved invaluable in this regard. The phosphatides are best observed on wet chromatograms under ultraviolet light (15). Under these conditions anionic phosphatides such as inositol phosphatide, phosphatidyl serine, and phosphatidic acid appear blue or purple, and the other phosphatides such as lecithin, sphingomyelin, lysolecithin, and phosphatidyl ethanolamine appear yellow or orange.

ANALYTICAL METHODS

Phosphorus analyses[5] were done as described previously (19). Nitrogen was determined by Nesslerization as follows: samples containing

[4] Other Rhodamine dyes were tested but were not effective in detecting the lipides on silicic acid impregnated paper. The smallest amount of lecithin, lysolecithin, and sphingomyelin which could be detected with Rhodamine G was 2–5 μg.

[5] It is absolutely necessary to use thoroughly clean acid-washed glassware in order to obtain reliable and reproducible analyses of P and N at the micro-level reported here. A Bausch and Lomb 'Spectronic' spectrophotometer was used in these experiments.

2–10 μg N were digested for 20 min. with ½ ml 70% perchloric acid (low gas flame). After cooling, to each tube were added 2 drops of a 2% solution of gum ghatti, 6 ml Nesslers reagent (20) and water to make a final volume of 10 ml. The solutions were allowed to stand for 15 min. and the colors were read at 420 mμ. This method gave quantitative conversion of amino-N (such as found in phosphatidyl ethanolamine, phosphatidyl serine, and sphingosine) to ammonia but did not give quantitative conversion of choline-N to ammonia. For the determination of N in phosphatides containing choline, calibration curves based on pure samples of lecithin and sphingomyelin were used rather than calibration curves based on ammonium sulfate.

Ester analyses were done by the method of Rapport and Alonzo (21). Aldehydes were determined with the Schiff's reagent as follows: samples of lipide containing 20–40 μg of aldehyde were dissolved in ½ ml of 90% acetic acid to which was added 1 drop of saturated aqueous mercuric chloride. The solutions were boiled for 30 sec., cooled, and to each were added 4 ml of Schiff's reagent[6] and the colors allowed to develop for 15 min. Since the Schiff's reaction product of long chain aldehydes forms a colloidal solution in water, it was found necessary to add 4 ml of t-butanol (Eastman Kodak, m.p. 24–24.5°) to each tube. The colors were read at 585 mμ exactly 2 min. after adding the t-butanol. Several alcohols were tested and t-butanol was found to give the lowest blank value. Aldehyde values were based on a standard calibration curve from n-tetradecaldehyde (Matheson Co., P 6017). Sugar-containing phosphatides were determined by the anthrone reagent (22) with galactose as the standard.

CHROMATOGRAPHY

Whatman no. 1 filter paper was impregnated with silicic acid essentially as described previously (15). However, in these experiments the sodium silicate solution was prepared as follows: 310 gm of silicic acid (Mallinckrodt, analytical reagent, 100 mesh, for chromatography) were slowly added to 1 liter of 7.2 N NaOH. After the silicic acid was completely dissolved, the solution was made to a volume of 1.7 liters with distilled water. For quantitative paper chromatography the impregnated papers were first washed for 2 hr. with running tap water and then washed thoroughly with distilled water (six 1-hour washes). The chromatographic solvent was diisobutyl ketone-acetic acid-water 40:25:5 (vol/vol). The chromatograms were run in cylinders measuring 6 in. internal diameter and 18 in. in height. Chromatography was carried out in a constant temperature room at 23°C. The time of the chromatographic run was 24–28 hours.

LOW TEMPERATURE CHROMATOGRAPHY

In order to minimize hydrolysis of the acetal phosphatides in solvents containing acetic acid, chromatography was also carried out at 0–5°C. In addition to the chambers mentioned above, excellent chromatograms were obtained by use of narrow 1000-ml graduate cylinders. For this latter chamber, paper strips measuring 4 cm wide were used. The following solvents were found to give the best separations at this lower temperature: diisobutyl ketone-acetic acid-water 40:20:3 and diisobutyl ketone-n-butyl ether-acetic acid-water 20:20:20:3 or 15:25:20:3 (vol/vol). The low temperature chromatography permitted for the first time the partial (and sometimes complete) separation of the acetal type phosphatidyl ethanolamine from the diester analog. It was found desirable to adjust the composition of the solvent system for phosphatide extracts of different tissues.

The studies reported in this paper were carried out on the phosphatides of pig heart ventricle. The heart muscle was extracted three times with chloroform-methanol 1:2 at 40°C. For the first extraction the heart muscle was homogenized in a Waring blendor with methanol and then the chloroform was added. Ten grams of wet heart muscle yielded 283 mg of total lipides which contained 3.0% P. For chromatography on paper the lipides were dissolved in isoamyl alcohol-benzene 1:1 at a concentration of 8.7 μg of lipide P per 10 μl of solution (290 μg of total lipides per 10 μl). A typical chromatogram is shown in figure 1. The various phosphatides were identified by characteristic spot tests (8) and where possible by comparing their mobility to those of authentic compounds. In addition, the individual phosphatide components were hydrolyzed and the liberated N-bases and phosphate esters determined qualitatively by paper chromatography. On the

[6] The Schiff's reagent was prepared by dissolving 1 gm of basic fuchsin in 100 ml of 1.0 N HCl, adding 5 gm of anhydrous sodium bisulfite, and making to 1 liter with distilled water. The solution is allowed to stand for 2 days before using.

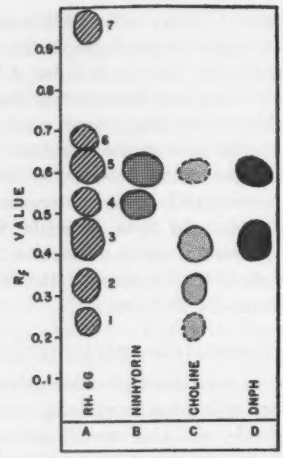


FIG. 1. Tracing of a chromatogram of total lipides of pig heart ventricle. Chromatography was carried out at 23°C on silicic acid-impregnated paper and employed diisobutyl ketone-acetic acid-water 40:25:5 as solvent (see text for details). *Rh.G.* = Rhodamine 6 G; *spots 1*, *4* and *6* gave a blue or purple color whereas the other spots gave a yellow color (as observed on wet chromatograms under ultraviolet light, 366 mμ.). The ninhydrin and choline reagents are described elsewhere (8). The positive choline test given by phosphatidyl ethanolamine (*spot 5*) is a non-specific test due to unsaturation. DNPH = 2,4,-dinitrophenylhydrazine. Chromatograms are either sprayed or dipped in a 0.15% solution of DNPH in 3 N aqueous HCl. Acetal phosphatides appear as orange spots. The identification of the phosphatides in each spot is given in the text.

basis of these combined tests *spot 1* is believed to be inositol phosphatide, but may contain a small amount of lysolecithin; *2* is sphingomyelin; *3* is lecithin (a mixture of the acetal and diester types as evidenced by the DNPH test); *4* is phosphatidyl serine; and *5* is phosphatidyl ethanolamine (a mixture of the acetal and diester types). *Component 6* has not yet been identified. *Spot 7* contains the nonphosphatides such as cholesterol, cholesterol esters, and free fatty acids.

QUANTITATIVE PAPER CHROMATOGRAPHY

The fine separations obtained by the aforementioned systems made possible the quantitative analysis of the phosphatides by paper chromatography and thus provided a new micromethod for the direct analysis of these compounds. The procedure is as follows: several chromatograms are run and the lipides detected by staining with Rhodamine 6G. The corresponding spots are cut off, combined, the area measured, and then the phosphatides are eluted by extracting three times for ½ hr. each under reflux with 0.8–1.0 N HCl in redistilled methanol. The extracts are filtered[7] into digestion tubes, concentrated to dryness and the lipide P is determined after digestion with perchloric acid. In this manner the quantitative distribution of the lipide P in each spot was ascertained. Control experiments with pure phosphatides were first performed in order to find the conditions for the quantitative recovery of the lipide P. The recovery of pure samples of lecithin and sphingomyelin was found to vary between 97 and 102%. It is necessary to determine the P value of the impregnated paper itself. This blank value was found to vary between 1.0 to 1.3 μg of P per 100 cm² of paper. The lipide P analyses were corrected by subtracting an appropriate blank value based on the area of the paper.

TABLE 1. QUANTITATIVE PAPER CHROMATOGRAPHY OF PIG HEART PHOSPHATIDES[a]

Frac.[b] No.	Lipide[c] P μg	% Total Lipide P	Phosphatide Components
0	1.70	1.97	Unidentified
1	1.49	1.72	Unidentified
2	8.21	9.50	Inositol phosphatide + some lysolecithin
3	8.45	9.90	Sphingomyelin
4	34.97	40.40	Lecithin—(diester and acetal types)
5	3.10	3.63	Phosphatidyl serine
6	18.24	21.10	Phosphatidyl ethanolamine—(diester and acetal types)
7	9.87	11.40	Unidentified
8	0.41	0.47	Unidentified

[a] Analysis done on 2.83 mg total pig heart ventricle lipides. Recovery of lipide P was 99.3%. Experimental details are given in the text. [b] Fractions are listed in order of increasing mobility of the respective phosphatides; 0 = origin material and 8 = material which ran near the solvent front. In reference to fig. 1, the fractions listed in this table correspond to the following spot numbers on the chromatograms: 2 = *spot 1*; 3 = *spot 2*; 4 = *spot 3*; 5 = *spot 4*; 6 = *spot 5*; 7 = *spot 6*; and 8 = *spot 7*. [c] P values have been corrected by subtracting an appropriate blank value (see text).

The data given in table 1 demonstrate the quantitative analysis of the pig heart phosphatides. Lecithin and phosphatidyl ethanolamine are the major phosphatides, and sphingomyelin,

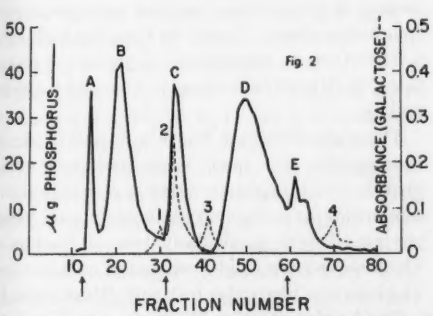
[7] The extracts were filtered through doubly acid-washed Whatman No. 44 filter paper.

phosphatidyl serine, inositol phosphatide, and the unidentified lipide no. 7 also occur in appreciable amount. The phosphatides listed above represent either the only or the major lipide in each spot. In some cases it is possible that more than one component may occur in a single spot.

QUANTITATIVE COLUMN CHROMATOGRAPHY

Although paper chromatography is admirably suited for work on a micro-scale, it does not easily permit the isolation of sufficient material for a more complete analysis. Hence, in order to further our investigations on the pig heart phosphatides, column chromatography on silicic acid was employed. The phosphatides were fractionated by solvents containing increasing amounts of methanol in chloroform. Although resolution of the phosphatides can be effected by use of various mixtures of diisobutyl ketone-acetic acid water, the much greater ease in removing chloroform and methanol by distillation at reduced pressure and moderately low temperature made these latter solvents more practical. In our hands prewashing the silicic acid with methanol-chloroform was important, since it gave more reproducible results and increased the adsorbing power of the silicic acid by substituting methanol for bound water (23). This latter treatment gave better results than activating the silicic acid by heating.

A typical elution curve of the phosphatides is shown in figure 2. Fractions 1–12 contained the non-phosphatides and were eluted with chloroform alone. The peak fractions A and B were eluted with 20% methanol in chloroform, C and D were eluted with 50% methanol in chloroform, and the remaining fractions were eluted with absolute methanol. Aldehyde-containing phosphatides were found only in fractions B and D. It is also evident that 4 different sugar-containing lipides were fractionated by this procedure. Weiss



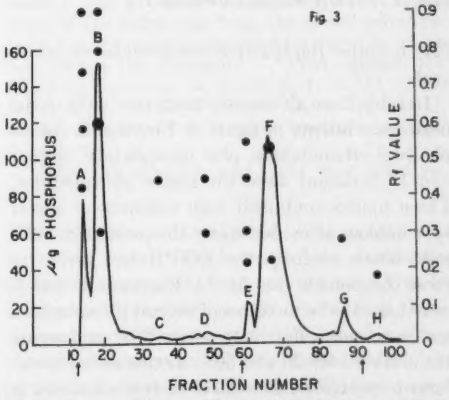


FIG. 2. Column chromatography of pig heart phosphatides on silicic acid. A total of 28.4 mg of pig heart lipides were dissolved in 3 ml chloroform and placed on a column (1.2 cm diameter) containing 10 gm silicic acid. The column was prepared by pouring a slurry of the silicic acid in petroleum ether into the column, allowing the adsorbent to settle, and then washing with 20 ml chloroform-methanol 1:1 followed by 25 ml chloroform. After applying the lipides to the column, fractionation was carried out with the following solvents in the order given: 50 ml chloroform, 100 ml chloroform-methanol 4:1, 150 ml chloroform-methanol 1:1, and 150 ml methanol. Fractions of 5-ml volume were taken. An air pressure head was maintained on the column to give a flow rate of about 1.0 ml/min. Solvent changes occurred at fractions 13, 32 and 60 as indicated by the upright arrows. Analysis of the fractions is given in table 2.

FIG. 3. Column chromatography of pig heart phosphatides on silicic acid. The column was prepared as described in figure 2. A total of 56.6 mg of lipide was fractionated on 10 gm silicic acid. Elution was carried out with the following solvents in the order given: 50 ml chloroform; 250 ml chloroform-methanol 4:1, 150 ml chloroform-methanol 1:1; and 100 ml methanol. Solvent changes occurred at fractions 13, 58 and 92 as indicated by upright arrows. Fractions of 5-ml volume were collected. A positive ninhydrin test was given by the 2 slower moving spots (Rf 0.31 and 0.60) in peak B and by the component with Rf value 0.31 in peak E. A positive test for choline was given by all the components in peaks F, G and H and by the fast moving spot in peak E. The two slower moving spots in peak A and the 2 fast moving spots in peak B were minor constituents. The 2 slower moving spots in peak B, the spots having Rf values 0.31 and 0.55 in peak E and all the components in peaks F, G, and H gave a yellow color with Rhodamine G. All the other spots gave either a blue or purple color with Rhodamine G. The analysis of the peak fractions is given in table 3.

TABLE 2. ANALYTICAL DATA OF FRACTIONS OBTAINED BY COLUMN CHROMATOGRAPHY OF PIG HEART LIPIDES[a]

Peak Fraction[d]	Lipide[b] P	% Total Lipide P	Spot Tests[c]			
			Ninhydrin	choline	Schiff's	anthrone
	μg					
A	49.7	5.81	−	−	−	−
B	236.7	27.7	+	+	+	−
C	135.3	15.8	+	−	−	+
D	341.7	40.0	−	+	+	−
E	86.2	10.1	−	+	−	−

[a] These analyses were done on the peak fractions given in fig. 2. [b] A total of 855 μg lipide P was put on the column and 851 μg were eluted. Recovery of P was 99.5%. [c] The choline test given by fraction B is a non-specific reaction due to unsaturated phosphatidyl ethanolamine (33). [d] Peaks include the following fractions: A, 13–16; B, 17–31; C, 32–42; D, 43–59; E, 60–85. *Peak B* was mainly phosphatidyl ethanolamine plus a small amount of phosphatidyl serine; *C* was a mixture of inositol phosphatide and other unidentified phosphatides, *D* was mainly lecithin, and *E* was a mixture of lecithin and sphingomyelin. *Peak A* was not identified.

(24) has also fractionated cerebrosides on silicic acid.

In table 2 are given some analytical data of the peak constituents in figure 2. Fraction B (phosphatidyl ethanolamine plus phosphatidyl serine) and D (lecithin) were the major phosphatides. These lipides contained high amounts of acetal type analogs as evidenced by the positive Schiff's test, which confirms the DNPH test given by these compounds (see fig. 1). Fractions C and E were found to be mixtures of several phosphatides and were more effectively resolved by prolonging the elution with 20 and 50% methanol in chloroform respectively. The effect of this alteration is shown in fig. 3.

The spots shown in figure 3 represent the phosphatide components of the single peak fractions only, and were determined by chromatography on silicic acid impregnated paper. As can be seen, some of the peak fractions are heterogeneous in composition. Indeed, the column chromatography concentrated some components and thereby made possible their detection. These minor constituents may escape detection on a chromatogram of the total lipide extract. Note that fraction C in figure 2 has been resolved into several components, indicated in figure 3 by peaks C, D and E. Furthermore, the sphingomyelin fraction G (fig. 3) has been resolved from the lecithin peak F and the small lysolecithin peak H. The components in fraction A remain unidentified but on the basis of their mobility and Rhodamine G test are believed to be acidic phosphatides. The large spot (Rf 0.60) in fraction B is phosphatidyl ethanolamine (a mixture of the diester and acetal types) and the slower moving component (Rf 0.31) is either a lysocephalin or a different kind of amino-containing phosphatide. The major faster moving component in fraction F is lecithin (a mixture of the diester and acetal types) whereas the minor slower moving spot is believed to be a different type of phosphatidyl choline.

Some analytical data of the peak fractions shown in figure 3 are presented in table 3. An inspection of the data reveals that fractions B (mainly phosphatidyl ethanolamine) and F (mainly lecithin) contain a high amount of acetal type phosphatides. This is evident from the molar ratios of ester/P and aldehyde/P and from the low fatty acid content (the theoretical fatty acid content for a diester phosphatide is about 70%. Particular attention is drawn to the unexpected high N/P ratio for the phosphatidyl ethanolamine fraction B. Hydrolysis studies showed that both serine and ethanolamine were present in this fraction but that sphingosine was completely absent. It may be that this high ratio is indicative of a phosphatide containing a dipeptide. Cheftel (25) has recently reported a similar finding.

The sugar/P ratio in fractions C and D (fig. 3) is suggestive of a novel sugar-containing phosphatide which might be either a phosphorylated cerebroside or a sugar-containing inositol phosphatide. This is particularly true of fraction C which moved as a single component on paper and which gave a blue color test with Rhodamine G.

The lecithin fraction F (fig. 3) was shown by hydrolysis with hot acetic acid to have a minimum of 40% of the acetal type phosphatidal choline. The lecithin was converted to the extent of 40% to lysolecithin with the concomitant production of free aldehyde. In this regard, Rapport and Alonzo (26) have shown that the acetal analog is a major constituent of beef heart lecithin. We have now isolated sufficient material to carry out an extensive study of the structure of the pig heart acetal type lecithin and have shown by a straightforward chemical method that the aldehyde is linked predominantly and possibly exclusively to the α-carbon atom of glycerol (27). This was done as follows: the lecithin was reduced under mild conditions with hydrogen to yield the ether derivative of the phosphatidal choline. Hydrolysis of this compound gave a long

chain ether of glycerol phosphate which on further prolonged hydrolysis yielded the glycerol ether. This latter compound on a molar basis consumed one mole of periodic acid with the concomitant liberation of a long chain aldehyde and a volatile aldehyde (HCHO). These findings extend the work of Klenk and Debuch (28) and Debuch (29).

In addition to the analyses shown in table 3, each peak fraction obtained by column chromatography was hydrolyzed with 1 N HCl for 1 hr. and the N-bases, phosphate esters and fatty acids which were liberated were determined qualitatively by paper chromatography. The N-bases were determined by chromatography on acetic acid-washed Whatman No. 1 paper with the following solvents: A-collidine-lutidine-1:1 saturated with water; B-phenol saturated with water; C-propanol-concentrated ammonia-water 60:30:10; and D-isopropyl ether-formic acid 90:60. The phosphate esters were also determined by solvents B, C and D. Fatty acids were determined by chromatography on Whatman No. 1 filter paper which was impregnated by dipping in a solution of 10% liquid petrolatum and using a solvent consisting of acetic acid-water 90:10 (saturated with petrolatum) (30).

Ethanolamine and serine were found in varying amount in fractions B, D and E. Sphingosine was found primarily in fraction G. Choline occurred in fractions F, G and H. The sphingomyelin fraction G was more refractory to hydrolysis than all the other lipides, and contained predominantly saturated fatty acids. The other fractions contained mainly unsaturated fatty acids some of which moved identically to linoleic and linolenic acids. The fatty acids in lecithin (peak F) were markedly different from those in phosphatidyl ethanolamine (peak B). Glycerophosphate occured in peaks B, E, F and H. Fraction G upon acid hydrolysis gave rise to two phosphate esters which are believed to correspond to sphingosine phosphate, sphingosinephosphorylcholine or phosphorylcholine. Some of the phosphate esters in fractions A, C, D and E were different from glycerophosphate and were not identified. It is very likely that one of these unidentified esters is an inositol phosphate.

LIPIDE ANALYSIS OF A PURIFIED CYTOCHROME PREPARATION OF PIG HEART

Similar experiments (both quantitative paper and column chromatography) were carried out on a purified cytochrome preparation of pig heart.

TABLE 3. ANALYTICAL DATA OF FRACTIONS OBTAINED BY COLUMN CHROMATOGRAPHY OF PIG HEART LIPIDES[a]

Peak Fraction[e]	Lipide[b] P	% Total Lipide P	Molar Ratio[d] Ester / P	Aldehyde / P	N / P	Sugar / P	Fatty Acid
	μg						%
A	141.7	8.35	1.40	*	0.68[c]	*	
B	549.3	32.30	1.00	0.96	2.13[c]	*	40.6
C	27.4	1.61	1.30	*		0.66	
D	73.6	4.34	1.20	*		0.55	
E	51.0	3.05					
F	738.6	43.50	1.30	0.88	1.09[c]	*	42.4
G	78.7	4.63	0.70	*	1.49		
H	25.0	1.47	1.20	*			

[a] These analyses were done on the peak fractions given in fig. 3. [b] A total of 1698 μg lipide P was put on the column and 1685 μg P were eluted. Recovery of P was 99.3% [c] The per cent N and P respectively in these fractions were as follows: A—0.78, 2.53; B—2.50, 2.60; F—1.66, 3.36. [d] Aldehyde/P ratios are approximations since it was assumed that the extinction coefficient of the aldehydes from the acetal phosphatides was the same as the tetradecaldehyde which was used as the standard. [e] Peaks include the following fractions: A, 14–16; B, 17–31; C, 32–40; D, 41–59; E, 60–62; F, 63–80; G, 81–92; and H, 93–113. Identification of peak fractions is given in text.

* Tests for aldehyde and sugar were negative.

This cytochrome preparation (31) contains a factor or factors which couple the enzymatic oxidation of succinate with cytochrome *c*. One of the aims of this study was to determine if there is a unique lipide pattern in the insoluble cytochromes and what role, if any, these lipides play in electron transport. The quantitative paper chromatographic analysis of the phosphatides of this preparation gave the following results (expressed as the percentage of total lipide P):[8] lysolecithin, 12.87; inositol phosphatide + lysocephalin, 10.59; lecithin (both the acetal and diester types), 29.30; phosphatidyl serine, 5.82; phosphatidyl ethanolamine (both the acetal and diester types), 18.01; an unidentified acidic phosphatide, 14.90; other unidentified phosphatides, 8.0. However, the phosphatide composition was found to be dependent on the manner of extraction of the lipides. When the lipides of the cytochrome preparation were extracted from the trichloracetic acid precipitated residue rather than by direct extraction of the preparation, the

[8] The lipides were obtained by direct extraction of the preparation with chloroform-methanol as described in the text.

lysolecithin value rose to 22.7% and the value for the unidentified acidic phosphatide dropped to 2.86%, whereas the values for the other constituents remained the same or changed very little. Hence, trichloracetic acid causes the hydrolysis of a labile lipide with the formation of lysolecithin. The high concentration of lysolecithin in this cytochrome preparation is indeed unique since this phosphatide is at most a trace constituent of normal tissues (16). Some of the lysolecithin and lysocephalin are very likely formed by enzymatic degradation during the preparation of the cytochrome. It is noteworthy that this chtochrome preparation was free from sphingomyelin and cerebrosides and its lipide composition differed markedly both from that of a purified pig heart cytochrome oxidase (32) and from the original heart muscle.

The results of these investigations give convincing evidence for the unlimited possibilities of chromatographic methods for the study of lipide problems. The quantitative paper chromatographic procedure reported here offers a new micro-analytical method as applied to the phosphatides and enables for the first time the direct analysis of these compounds in a complex mixture.

The authors acknowledge the contributions of Dr. Claude Reed of the Department of Hematology of this University on the study of conditions for the quantitative elution of known pure phosphatides from silicic acid impregnated paper. The authors are also indebted to Dr. Elmer Stotz for his constant aid and advice.

ADDENDUM

After completion of this manuscript two papers appeared by Rapport and co-workers (34, 35) on the structure of plasmalogens claiming by an enzymatic method that the aldehyde group is linked to the β-carbon atom of glycerol in the beef heart phosphatidal choline. This report differs from both our findings and those of Klenk and Debuch (28). It may be that there exists a species difference in these phosphatides or that the specificity and mechanism of action of lecithinase A requires further study.

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DISCUSSION: CEPHALIN SEPARATIONS

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PAPER chromatography, especially it would appear on silicic acid impregnated paper, has become a useful tool as a check on the presence of certain lipide components in tissues and of their appearance and disappearance during metabolic studies. In certain specific cases it has a definite advantage over silicic acid column chromatography, in that it will separate phospholipides which are not easily separated on columns. For example, Rhodes and Lea (1) found that while lysophosphatidylethanolamine and lecithin had widely differing R_f's on silicic acid impregnated papers run in 4:1 $CHCl_3$:MeOH, i.e., 0.44 and 0.63, respectively, they were eluted together by 4:1 $CHCl_3$:MeOH on a silicic acid column. Similarly, the phosphoglyceracetals have not so far been separated from their corresponding phosphoglycerides on silicic acid columns. Thus, Rapport and Alonzo (2) found that the ethanolamine plasmalogen and phosphatidylethanolamine of lymphosarcoma were eluted together by approximately 30% ethanol in hexane, while Hanahan (3) has found that the amino phosphatides and lecithin of beef heart mitochondria and electron transport system eluted by 4:1 and 3:2 $CHCl_3$:MeOH, respectively, both contained Schiff reacting material; while, interestingly enough, the inositol lipide which is eluted with 3:2 $CHCl_3$:MeOH as a distinct peak before the lecithin, contained no aldehyde. Macfarlane and Gray (4) also report that phosphoglyceracetals from beef heart are eluted with 4:1 and 3:2 $CHCl_3$:MeOH together with their corresponding phosphoglycerides. Marinetti *et al.* (5) have obtained good separation of acetal phospholipides away from other components on paper chromatograms, but their solvent systems are not readily applicable to column chromatography. Skipski and Rapport (6) have also obtained further separations of silicic acid column peaks by refractionation on silicic acid impregnated papers. Precautions were necessary to avoid hydrolysis of acid sensitive plasmalogens. Lovern *et al.* (7) found appreciable liberation of free aldehyde fram ram sperm phosphoglyceracetals if they were allowed to remain on silicic acid columns for more than 24 hr.

Rhodes and Lea (8), Hanahan *et al.* (9), Rapport and Alonzo (2), Kates (10) and Marinetti (11) have now worked out suitable solvent systems for the separation of the amine-phospholipides, the choline-containing phospholipides, including sphingemyelin and the inositol-monophosphate lipides on silicic acid columns. Amongst the known phospholipide classes the challenge therefore remains to separate the phosphoglyceracetals from their corresponding phosphoglycerides and the serine from the ethanolamine-containing lipides. The inositol-diphosphate lipide from brain is also difficult to separate from serine-containing phospholipide. Hanahan *et al.* (9) have found that serine- and ethanolamine-containing phospholipides are often eluted as a sharp peak with 4:1 or 7:1 $CHCl_3$:MeOH, though a small peak preceding the main peak is usually predominantly serine-containing. Hawthorne and Dils (12) found that chromatography of a cephalin fraction gave two peaks, both of which contained serine and ethanolamine. Hydrogenation considerably sharpened the peaks, but still did not give pure fractions. The influence of the nature of the fatty acids on the separation of the phospholipides is shown by the different solvent systems required for rat, beef and salmon liver cephalins. The aminephosphatides, inositolmonophosphatides, lecithin and sphingomyelin of rat and beef liver can be adequately separated by successive use of 4:1, 3:2 and 1:4 $CHCl_3$:MeOH (9). The phospholipides of salmon liver which are present in approximately the same proportions, but probably contain up to 70% $C_{20} + C_{22}$ highly unsaturated fatty acids (13), require 5:1, 1:1, and 1:6 $CHCl_3$:MeOH to separate them effectively (14).

There would appear to be four ways of tackling the problem of lipides which do not separate on chromatography: *1*) altering the lipides as by, for example, acetylation; *2*) altering the silicic acid; (Dr. Skipski tells me that pyridine pretreatment of the silicic acid can even invert the elution of phosphatidyl ethanolamine and serine); *3*) changing the solvent systems; and *4*) rechromatography of individual peaks either with the same or different solvent systems. Rechromatography of individual peaks is in fact often useful for further purification. Hanahan (15) has shown in a previous paper how an inositolmonophosphatide could be further purified by rechromatography of the original inositol-containing peak. Success in these separations is entirely empirical, depending on the loading of the column, choice of eluting solvent, and time of changing the eluting solvent. Changing the solvent too soon may result in a sharp peak which has no chemical significance. Lovern (16) found that rechromatography of the non-phosphorus containing lipides of the flesh of the cod was useful. Each original peak could be separated into 3 or 4 components and each fraction

contained traces of certain of the preceding fractions and also fractions, the main part of which would be eluted later with a more polar solvent. While these effects are not so important in analytical work in radioactive tracer studies, they could produce serious errors. Work in Dr. Hanahan's laboratory has shown that rechromatography of the serine and ethanolamine glycerophosphatide peak ($CHCl_3$:MeOH 7:1) of beef liver gave at least 3 peaks. However, analyses of the aqueous portion of the hydrolysates of these peaks by DNFB (17) indicated that they all appeared to be similar mixtures.

This brings me to the point of the actual analyses of the phospholipide peaks. It is simple to take a tissue and analyze for choline, serine, ethanolamine, etc., and quote phospholipide values for a tissue. Many workers do not even compare their analyses with a total nitrogen on their lipide hydrolysates. It is however entirely a different matter to prove that a particular chromatographic peak is in fact a pure compound. This is a reason why I feel that not too much emphasis should be laid on paper chromatography. It is necessary to have enough material to do a thorough quantitative analysis, and it is just these minor components which we know least about and want to study which give the most equivocal spots on paper chromatography.

And therefore, here I feel it would be relevant to discuss some of the errors in the methods of lipide analyses. Dittmer (18) has made a detailed study of some of the methods available for serine and ethanolamine and has found some serious inherent errors. For example, although it is known that aqueous 6 N HCl hydrolysis causes losses of at least 10% of the lipide nitrogen (19) and alkali up to 60% (20) as ammonia, many workers continue to use them rather than the less destructive dry methanolic HCl. Any ammonia formed causes errors in the DNFB method. DNP-amine partitions between DNP-serine and DNP-ethanolamine, giving erroneously high results to both fractions. Methanolic HCl hydrolysis, however, causes formation of methylserine which can only be quantitatively decomposed to free serine by refluxing in slightly alkaline aqueous solution (pH 8). Permutit and Zeokarb 215 have both been found unsatisfactory for separation of serine and ethanolamine. Unless sufficient ethanolamine or other substance of higher pK than serine, as for example choline, are present to saturate the resin, serine is also partially absorbed. This characteristic varies from batch to batch of resin. A batch of IRC 50 has been found extremely satisfactory in Dr. Hanahan's laboratory for the separation of serine and ethanolamine, but this does not mean that the resin should not be checked by other workers. The periodate oxidation of serine and ethanolamine (20) should be done in the cold, the excess periodate destroyed and then the ammonia distilled. Choline has been found to react with periodate at 100° at pH 9, to give a distillable base. A beef liver serine and ethanolamine phospholipide fraction (9) was found on the basis of periodate oxidation of an hydrolysate to contain 50% of a nitrogen containing substance, X, resistant to periodate. From the amount of glycerophosphate present, the parent compound appeared to be a glycerophosphatide. On hydrolysis of the lipide, X gave a DNP-derivative and partitioned with the DNP-ethanolamine and was thus not detected by this method. Similarly, it ran with ethanolamine on paper chromatograms in two solvents and stained with ninhydrin but not with the phosphomolybdate reagent. This compound may be a decomposition product as the beef liver phospholipide had been stored in $CHCl_3$ for 4 months at −25°. It is interesting to note, however, that Lea (21) found that old samples of synthetic phosphatidylethanolamine gave evidence of decomposing to a compound the nitrogenous moiety of which did not react with DNFB. GPX would therefore appear to be a new phospholipide.

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BIOSYNTHESIS OF PHOSPHOLIPIDES[1]

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THE experiments of Friedkin and Lehninger (1) in 1948 provided the first clear-cut demonstration of the synthesis of phospholipides in a cell-free enzyme system. In recent years, there has been considerable progress in working out the network of enzymatic reactions leading to the biosynthesis of phospholipides from simple precursors. A rather complete pattern of reactions accounting for the biosynthesis of a typical phospholipide such as lecithin is shown in figure 1. Each of these reactions has now been demonstrated in cell-free enzyme systems and the intermediates have been well characterized chemically. Further, each reaction has been shown to lead to the net synthesis of product, a point which is of importance since many of the reactions have been followed by the isotopic tracer technique and it is therefore essential to rule out the occurrence of exchange reactions leading to isotope incorporation without real synthesis.

An essential feature of the scheme presented in figure 1 is the intermediary formation of a nucleotide form of choline, cytidine diphosphate choline, the structure of which is shown in figure 2. An analogous set of reactions involving phosphorylethanolamine and cytidine diphosphate ethanolamine is also known and leads to the formation of phosphatidylethanolamine.

Although the reactions shown were worked out mostly with liver enzymes, the independent work of Rossiter and his collaborators, described in this Symposium, indicates that the biosynthesis of phospholipides in brain tissue follows the same pattern.

As shown in figure 1, CDP-choline[2] reacts with a D-1,2-diglyceride to form lecithin (2, 3). Alternatively, the D-1,2-diglyceride may react with long-chain thioesters of CoA to form triglycerides (4). A close interconnection between the biosynthesis of phospholipides and triglycerides is thus apparent, the significance of which will be considered later in this discussion.

Role of L-α-glycerophosphate. In 1953, Kennedy (5) showed that α-glycerophosphate is an obligate intermediate in the incorporation of P_i^{32} into a phospholipide having the properties of a phosphatidic acid, using particulate enzyme preparations from rat liver which were also carrying on oxidative phosphorylation. The addition of free glycerol was found to double the rate of incorporation, suggesting that the phosphorylation of glycerol was the first step in the conversion of glycerol to phospholipide. The phosphorylation of glycerol was found to be catalyzed by an enzyme (glycerokinase) which was extensively purified by Bublitz and Kennedy (6) who were also able to show the direct effect of the purified enzyme in stimulating the formation of radioactive phosphatidic acid. These findings have been confirmed more recently by Marinetti *et al.* (7).

Synthesis of Phosphatidic Acids. In an important contribution, Kornberg and Pricer (8) showed that L-α-glycerophosphate is esterified in a reaction with long chain thioesters of CoA, catalyzed by a particulate enzyme from guinea pig liver. No direct esterification of free glycerol could be detected in these studies.

Role of Phosphatidic Acids. Although in the experiments of Kornberg and Pricer (8) and of Kennedy (5) a very rapid synthesis of phosphatidic acids *in vitro* was noted, phosphatidic acids have not yet been demonstrated to be normally occurring constituents of mammalian tissues. Marinetti and Stotz (9) were unable to detect phosphatidic acids in rat heart or liver, nor was Dawson (10) able to show the presence of these compounds in tissues of the guinea pig.

This apparent anomaly led Marinetti and

[1] Experimental work in the author's laboratory on the enzymatic synthesis of phospholipids and triglycerides has been supported by grants from the Nutrition Foundation, the Life Insurance Medical Fesearch Fund, and the United States Public Health Service (B-1199).

[2] The following abbreviations are used: Adenosine triphosphate, ATP; cytidine mono- and triphosphate, CMP and CTP; guanosine diphosphate, GDP; inosine triphosphate; ITP; uridine triphosphate, UTP; cytidine diphosphate choline, CDP-choline; uridine diphosphate choline, UDP-choline; guanosine diphosphate choline, GDP-choline; adenosine diphosphate choline, ADP-choline; cytidine diphosphate ethanolamine, CDP-ethanolamine; coenzyme A, CoA; orthophosphate, Pi; inorganic pyrophosphate, P-P.

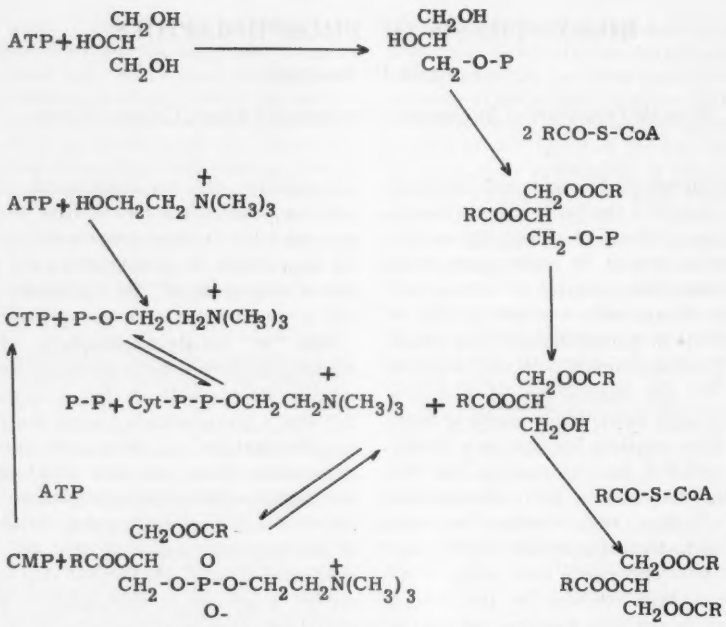

FIG. 1. Enzymatic synthesis of phospholipides and triglycerides.

Stotz (9) to question the significance of the known enzymatic reactions leading to the synthesis of phosphatidic acids and to suggest that the pathways of phospholipide biosynthesis *in vivo* and *in vitro* may be different. However, if phosphatidic acids are intermediates in the biosynthesis of phospholipides rather than end-products, then the stationary concentration *in vivo* may be quite low, since they may be further metabolized as rapidly as they are formed.

The discovery of an enzyme in chicken liver and in various tissues of the rat which cleaves phosphatidic acids with the formation of P_i and D-1,2-diglyceride (11, 12) offers an explanation for the failure of phosphatidic acids to accumulate. The enzyme from chicken liver appears to be rather specific for phosphatidic acid (table 1). The accumulation of phosphatidic acids *in vitro* in the experiments of Kornberg and Pricer (8) and of Kennedy (5) may be attributed in part at least to the fact that magnesium ions were added to the enzyme incubation mixtures used by these workers. Magnesium is an effective inhibitor of phosphatidic acid phosphatase (11).

These results suggest that the function of phosphatidic acid is to act as precursors of D-1,2-diglycerides. Since D-1,2-diglycerides are in turn precursors of both phospholipides and triglycerides, it is clear that phosphatidic acids must be regarded as extremely important intermediates in lipide metabolism. The direct esterification of free glycerol by long chain thioesters of CoA has not yet been observed, although such a reaction has been sought by Kornberg and Pricer (8), Weiss and Kennedy (13), and Stein and Shapiro (14) under conditions in which the esterification of L-α-glycerophosphate readily occurred. Although it is notoriously unwise to place too much weight on the failure to find a given enzymatic reaction, it is nevertheless true that at present the pathway over L-α-glycerophosphate and phosphatidic acid is the only known route for the incorporation of free glycerol into lipides.

Phosphorylation of Choline. A kinase catalyzing the phosphorylation of choline by ATP was found in liver and in yeast by Wittenberg and Kornberg (15), who isolated and partially purified the enzyme from yeast. Although the isolation of an ethanolamine kinase has not yet been reported, it seems reasonable to suppose that the phosphorylation of ethanolamine takes place by an essentially similar reaction.

Function of Cytidine Coenzymes in Biosynthesis of Phospholipides. In an investigation of the mechanism of incorporation of phosphoryl-

choline into lecithin, a reaction first observed by Kornberg and Pricer (16), Kennedy and Weiss (2) found that the incorporation was greatly stimulated by amorphous preparations of ATP, whereas crystalline ATP was completely inactive (table 2). A closer investigation of the cofactor requirement disclosed that the true cofactor is cytidine triphosphate, present in trace amounts in the amorphous ATP preparations. The requirement for CTP is highly specific; it cannot be replaced by any other nucleotide of a large number tested.

The discovery of the requirement for CTP for the conversion of phosphorylcholine to lecithin is the first demonstration of a specific function of a cytidine nucleotide as a coenzyme for a metabolic reaction of obvious importance. It has now been firmly established that the mode of action of CTP in this system involves the transfer of the cytidyl portion of CTP to phosphorylcholine with the formation of a novel coenzyme form of choline, CDP-choline (3).

Synthesis of CDP-choline, CDP-ethanolamine and Related Compounds. The chemical procedures developed by Khorana (17) have been applied by Kennedy (18) to the synthesis of CDP-choline, CDP-ethanolamine, ADP-choline, UDP-choline and GDP-choline. These syntheses were accomplished by the use of N,N′-dicyclohexylcarbodiimide for the condensation of the phosphorylated bases and the appropriate nucleotide:

$$\text{Cyt-P} + \text{P-choline} + \text{RN}{=}\text{C}{=}\text{NR} \rightarrow$$

$$\text{Cyt-P-P-choline} + \text{RNHCONHR}$$

The products of the reaction were separated and purified by chromatography on ion exchange resins.

When tested in the isolated enzyme systems, both CDP-choline and CDP-ethanolamine displayed the properties required of obligate intermediates in the synthesis of lecithin and phosphatidylethanolamine respectively. Thus CDP-choline acted as a much more efficient precursor of labeled lecithin than equal amounts of CTP + P-choline (3). Furthermore, when labeled P-choline was tested in the presence of unlabeled CDP-choline, the CDP-choline acted as an effective isotope diluting agent.

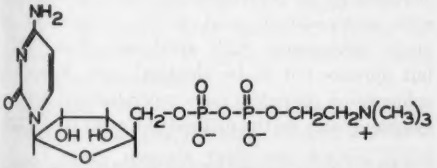

FIG. 2. Structure of cytidine diphosphate choline.

TABLE 1. SPECIFICITY OF PHOSPHATIDIC ACID PHOSPHATASE FROM CHICKEN LIVER

Substrate	P_i Formed, μM
Lecithin	0.00
Phosphatidic acid	0.71
DL α-glycerophosphate	0.06
β-glycerophosphate	0.10
Glucose-6-phosphate	0.05

Each tube contained 60 μM of maleate buffer of pH 6.33 and 0.1 ml of enzyme in final volume of 1.0 ml; 2.0 μM of each substrate were added as shown. The tubes were incubated for 30 min. at 37° and were stopped by the addition of 5 ml 10% trichloracetic acid, and P_i determinations were made on aliquots of the supernatant solution after centrifugation. Values shown are corrected for small blank values obtained in control tubes which were incubated without substrate.

PC- and PE-Cytidyl Transferases. The names phosphorylcholine-cytidyl transferase (PC-cytidyl transferase) and phosphorylethanolamine-cytidyl transferase (PE-cytidyl transferase) have been suggested for the enzymes which catalyze the synthesis of CDP-choline and CDP-ethanolamine from CTP and the corresponding phosphorylated base.

PC-cytidyl transferase is widely distributed in nature, being found in yeast and carrot root as well as in various tissues of the rat, hog and guinea pig. The PC-cytidyl transferase of guinea pig liver has been extensively studied by Borkenhagen and Kennedy (19). It is rather stable to heating at 55°C, requires magnesium or manganese ions and is firmly bound to intracellular particles. Its complete specificity for CTP is shown in table 3.

In contrast to PC-cytidyl transferase, PE-cytidyl transferase is readily obtained in soluble form and is labile to heating at 55°. The properties of the PE enzyme are currently being studied.

PC-Glyceride Transferase. The reaction between CDP-choline and D-1,2-diglyceride is catalyzed by an enzyme for which the name PC-glyceride transferase has been proposed. The first attempts to identify the lipide acceptor which reacts with CDP-choline were unsuccessful, because of the technical difficulty encountered in adding water-insoluble lipide substances to the enzyme system in a form in which they can penetrate to the enzyme surface. It was finally

found that D-1,2-diglycerides containing at least one unsaturated fatty acid residue and suitably emulsified in 'Tween-20' (polyoxyethylene sorbitan monolaurate; Atlas Powder Co.) greatly stimulate the conversion of CDP-choline to lecithin. It now became possible to demonstrate for the first time an actual net synthesis of phospholipide *in vitro* (table 4). It is seen that there is an almost complete requirement for added diglyceride both for incorporation of radioactivity from labeled CDP-choline and for net synthesis of lecithin.

The PC-glyceride transferase is quite specific for 1,2-diglycerides. Lecithin, phosphatidic acid, triglyceride, or 1,3-diglyceride, cannot be substituted. It is completely specific for CDP-choline; when chemically synthesized UDP-choline, GDP-choline and ADP-choline were tested, no significant activity was detected (table 5). The same high degree of specificity is noted with enzyme preparations from yeast and from rat liver, suggesting that the same fundamental mechanisms are involved in synthesis of phospholipides in very different types of living cells.

It is of interest that the PC-glyceride transferase reaction is readily reversible. When CMP labeled with P^{32} was incubated with the enzyme, labeled CDP-choline was formed by 'cytidylolysis' of the lecithin present in the enzyme particles (table 6).

The CMP formed as a product of the PC-glyceride transferase reaction can be rephosphorylated to CTP at the expense of ATP as shown by Herbert and Potter (20). These reactions complete a cycle in which the CTP is regenerated to react with another mole of phosphorylcholine in a true catalytic fashion (fig. 1). At each turn of this cycle one mole of phosphorylcholine is converted to the coenzyme form and subsequently to lecithin.

TABLE 2. COFACTOR REQUIREMENT FOR INCORPORATION OF P-CHOLINE-p-^{32} INTO LECITHIN

Cofactor Added	P-choline Incorporated into Lecithin (μM)
5 μM amorphous ATP (lot 116)	5.1
5 μM crystalline ATP (lot 122)	0.2
5 μM ATP (lot 122) + 0.5 μM ITP	0.0
5 μM ATP (lot 122) + 0.5 μM UTP	0.4
5 μM ATP (lot 122) + 0.5 μM GDP	0.4
5 μM ATP (lot 122) + 0.5 μM CTP	15.0

Each tube contained 10 μM $MgCl_2$, 50 μM of phosphate buffer of pH 7.4, 3 μM of P-choline-P^{32} (113,500 c.p.m./μM) and 0.25 mg. of a 10% suspension of lyophilized rat liver particles in a final volume of 1.0 ml. Other cofactors were added as indicated. The tubes were incubated for 1 hr. at 37°. From Kennedy and Weiss (2).

TABLE 3. NUCLEOTIDE SPECIFICITY OF PC-CYTIDYL TRANSFERASE

Nucleotide Added	P-Choline Converted to Nucleotide (μM)
Exp. 1	
0.20 μM CMP	0
0.20 μM CDP	0
0.20 μM CTP	45
Exp. 2	
0.50 μM CTP	78
0.50 μM ATP	0
0.50 μM GTP	0
0.50 μM UTP	0
0.50 μM ITP	0

Each tube contained 10 μM $MgCl_2$, 8 μM P-choline-P^{32}, 20 μM KF, 50 μM Tris buffer of pH 7.5, 10 μM cysteine and 0.20 ml enzyme. The final volume was 1.0 ml. The added nucleotide was varied as shown. The tubes were incubated at 37° for 1 hr.

Enzymatic Synthesis of Sphingomyelin. Since both sphingomyelin and lecithin are phospholipides containing a similar phosphorylcholine moiety and a monophosphate diester linkage, the possibility was suggested (3) that CDP-choline might be involved in the biosynthesis of sphingomyelin. By analogy with the PC-glyceride transferase reaction, the following synthetic scheme might be postulated:

N-acylsphingosine + CDP-choline

$$\rightleftharpoons \text{Sphingomyelin} + \text{CMP}$$

Evidence supporting such a formulation has recently been obtained by Sribney and Kennedy (21). These workers have found an enzyme in chicken liver which catalyzes an extensive net synthesis of a phospholipide having the properties of sphingomyelin when supplemented with CDP-choline and 'acetyl sphingosine.' As much as 25–30 mg of the enzymatically synthesized phospholipide can be isolated by chromatography on silicic acid. The identity of 'acetyl sphingosine' is not yet certain;[3] it is a product obtained by the treatment of crude sphingosine with acetic-sulfuric acid or by acetylation of crude sphingosine with acetic-anhydride (22), but appears not to be identical with N-acetylsphingosine prepared from recrystallized triacetylsphingosine by the method of Carter *et al.* (22).

[3] This 'active ceramide' has now been identified as N-acetyl-*threo-trans*-sphingosine.

TABLE 4. NET SYNTHESIS OF LECITHIN IN AN ISOLATED ENZYME SYSTEM

Additions	Lipid P* (Micro-atoms)	Δ Lipid P* (Micro-atoms)	Total Counts Incorporated
None	1.19		
CDP-choline	1.23	0.04	960
D-1,2-diglyceride	1.17	0	
CDP-choline + D-1,2-diglyceride	2.25	1.06	13,375

Each tube contained 20 μM $MgCl_2$, 10 μM cysteine, 40 μM Tris buffer of pH 7.4, 2 mg 'Tween-20' and 8 mg chicken liver particles in a final volume of 1.0 ml. 3 μM of CDP-choline labeled with choline-1,2-C^{14} were added as indicated. Tubes were incubated at 37° for 2 hr. From Weiss, Smith and Kennedy (11).

* Values in microatoms.

Occurrence of Cytidine Nucleotides in Nature. CDP-choline and CDP-ethanolamine have been isolated from the liver of the rat and the hen, and the concentrations in these tissues measured quantitatively by the isotope dilution technique (3). These nucleotides have also been found in yeast (3) and in cat brain (13). The isolation of the sodium salt of CDP-choline in crystalline form from yeast has been reported by Lieberman, Berger and Giminez (23). The widespread occurrence of CDP-choline and CDP-ethanolamine in nature strongly supports the view that these compounds are intermediates in biosynthesis *in vivo* as well as in the isolated enzyme systems.

Baddiley and coworkers have recently identified two nucleotides from *Lactobacillus arabinosus* as cytidine diphosphate glycerol and cytidine diphosphate ribitol (24, 25). The glycerol compound in particular suggests a function of these compounds in the realm of lipide metabolism.

The brilliant work of Leloir and his collaborators has led to the recognition of the importance of uridine coenzymes in carbohydrate metabolism. It is possible that further work may disclose a series of cytidine compounds of comparable and highly specific function in lipide metabolism. It has been suggested (3) that any adequate understanding of the complex functions of ribonucleic acid must account for the fact that all four nucleotides found in ribonucleic acid have coenzyme function, and that there appears to be a curious 'division of labor' between cytidine and uridine compounds in lipide and carbohydrate metabolism respectively.

Common Pathways for Biosynthesis of Phospholipides and Triglycerides. Despite extensive efforts, until recently little was known about the mode of biosynthesis of triglycerides. The theory that triglycerides might be synthesized by reversal of lipase action has been much discussed in the literature. Borgstrom (26) has shown that the incorporation of free fatty acids into glycerides may be catalyzed by lipase. Further support for this hypothesis may also be found in experiments by Jedeikin and Weinhouse (27) from which it appears that the incorporation of labeled palmitic acid into neutral fat by liver slices may not require a coupled energy source.

Tietz and Shapiro (28) were the first to show the operation of a synthetic pathway clearly different from the reversal of lipase action. These workers found that the incorporation of labeled fatty acid into neutral fat in liver homogenates required ATP and some heat stable cofactor.

The fact that phosphatidic acids may be enzymatically dephosphorylated to yield D-1,2-diglycerides and that D-1,2-diglycerides are precursors of phospholipides suggested these compounds might also be involved in the biosynthesis of triglycerides. In confirmation of this hypothesis, Weiss and Kennedy (3) found that a particulate enzyme from chicken liver catalyzed a reaction between D-1,2-diglycerides and palmitoyl CoA with the net formation of triglyceride (table 7). As is the case with the PC-glyceride transferase reaction, it is necessary to emulsify the diglyceride with a surface-active agent in order to penetrate to the enzyme surface.

A recent note by Stein and Shapiro (14) offers strong support for the formulation of triglyceride synthesis shown in figure 1. It has been found that the heat stable cofactor described in the previous experiments of Tietz and Shapiro (28)

TABLE 5. NUCLEOTIDE SPECIFICITY OF PC-GLYCERIDE TRANSFERASE OF RAT LIVER AND OF YEAST

	P-choline Incorporated into Lecithin (μM)	
	Liver	Yeast
UDP-choline	0.7	0.3
GDP-choline	0.0	0.0
ADP-choline	0.0	0.0
CDP-choline	64.8	84.1

Each tube contained 40 μM $MgCl_2$, 100 μM Tris buffer pH 7.4, 20 μM cysteine, 100 μM KF and 0.6 ml rat liver mitochondria or crude dialyzed autolysate of brewer's yeast as indicated. 0.20 μM of labeled nucleotide of identical specific activity (50,000 cpm/μM) was added as shown. Tubes were incubated for 1 hr. at 37°. From Kennedy and Weiss (3).

may be replaced by α-glycerophosphate + CoA. It is significant that free glycerol or β-glycerophosphate cannot be substituted for α-glycerophosphate. The requirement for ATP, CoA and α-glycerophosphate rather than free glycerol may be taken to indicate that the pathway of glycerol into triglyceride in these experiments is over phosphatidic acid and D-1,2-diglyceride as intermediates. This interpretation is further supported by experiments in which Stein and Shapiro (13) have shown that glycerol-labeled α-glycerophosphate is effectively converted to neutral fat.

Physiological Significance of Studies With Cell-free Enzyme Systems. The present era of biochemistry has seen a rapid and dramatic expansion of our knowledge of biosynthetic processes in general. As has been the case in other fields, little information could be obtained as to the detailed mechanism of the biosynthesis of phospholipides and triglyceride by experiments with intact animals. Such information can be derived only from experiments with isolated enzymes. While the necessity and value of such experiments cannot be doubted, the question always remains as to what extent the reactions observed *in vitro* reflect what is going on in the intact animal. It is for this reason that emphasis has been placed upon the widespread occurrence in nature of CDP-choline and CDP-ethanolamine, which is good evidence for the function of these compounds in the living cell as well as in isolated enzyme systems.

The demonstration of the reactions shown in figure 1 of course does not preclude the existence of alternative reactions *in vivo* leading to the formation of the same intermediates or products. To consider one obvious example, it is clear that L-α-glycerophosphate may be produced not only by the phosphorylation of glycerol catalyzed by glycerokinase, but also from triose phosphate arising during glycolysis.

The reaction pattern shown in figure 1 discloses an intimate interconnection between the synthesis of triglycerides and phospholipides. If the formulation shown does in fact represent a quantitatively important metabolic pathway *in vivo*, the physiological significance of this interconnection deserves considerable thought and attention. For example, no satisfactory explanation of the well-known lipotropic action of choline is as yet available. Experiments *in vivo* based on the known enzymatic reactions leading to the synthesis of phospholipides and triglycerides might shed some light on this important problem in physiology.

TABLE 6. REVERSIBILITY OF LECITHIN SYNTHESIS

System	Counts Recovered as CDP-choline
CMP^{32} + lecithin	52,000
P^{32}-choline + CMP	0

Experimental conditions were similar to those described in table 4, except that no diglyceride was added.

TABLE 7. ENZYMATIC NET SYNTHESIS OF TRIGLYCERIDES

System	Radioctivity of Triglyceride	Total Triglyceride (μM)
Palmitoyl-S-CoA + D-1,2-diglyceride	210,000	2.46
Palmitoyl-S-CoA + D-1,2-diglyceride, boiled enzyme	610	.72
D-1,2-diglyceride omitted	12,200	.79
Pamitate + CoA in place of palmitoyl-S-CoA	3,350	.61

The complete system contained 1000 μM Tris buffer of pH 7.4, 60 μM $MgCl_2$, 40 μM D-1,2-diglyceride were indicated, 4 μM of palmitoyl-S-CoA labeled with palmitate-1-C^{14} (140,000 cpm/μM), 20 mg 'Tween-20,' 80 μM cysteine and 1.0 ml. of a suspension of particles from chicken liver in final volume of 10 ml. Tubes were incubated for 2 hr. at 40°. From Weiss and Kennedy (4).

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DISCUSSION: BIOSYNTHESIS OF PHOSPHATIDES IN BRAIN AND NERVE[1]

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THE synthesis of phosphatides in brain and nerve is of considerable interest in relation to the formation of myelin and the problem of demyelination. Recently Rossiter, Pritchard and Strickland (1) summarized experiments indicating that the radioactivity from a variety of C^{14}-labeled precursors is readily incorporated *in vitro* into the glycerophosphatide fraction of brain and nerve preparations. The results suggest that in these tissues phosphatides are formed *in situ* from small molecules.

The labeling of the glycerophosphatides may be represented as shown in figure 1. The fatty acid moiety of the glycerophosphatide molecule can be labeled from acetate, the glycerol moiety from glycerol, and not from the bases, and the base moiety can be labeled from the appropriate base, as such, and not from glycerol. Kennedy and Weiss (2) demonstrated that in liver tissue the phosphate-base is incorporated into the glycerophosphatide as a unit. As will be seen below, the same is true for brain and nerve.

WALLERIAN DEGENERATION

At the present time there is much evidence derived from electron microscopic (3) and other studies that in peripheral nerve the Schwann cells are responsible for the production of myelin. If this is so, it is of great interest, and importance, to study the ability of a nerve to form phosphatide in situations where the population of Schwann cells is increased.

If a peripheral nerve is sectioned, there is a proliferation of Schwann cells in that part of the nerve distal to the point of section. The cellular proliferation forms part of the classical series of changes known as Wallerian degeneration. Rossiter, Pritchard and Strickland (1) showed that when fragments of cat sciatic nerves, sectioned some days previously, were incubated *in vitro* with radioactive precursors, such as inorganic P^{32}, acetate-1-C^{14}, glycerol-1-C^{14}, choline-1,2-C^{14}, ethanolamine-1,2-C^{14}, or serine-3-C^{14}, the specific activity of the phosphatide of the degenerating nerve was considerably greater than that of non-sectioned control nerves.

It is suggested that this increase in the labeling of the phosphatides represents an increase in the potentiality of the nerve for remyelination. This increase is associated with the increase in the population of Schwann cells.

BIOSYNTHESIS OF LECITHIN IN BRAIN

Largely as a result of the work of Kennedy (2, 4), much is known of the metabolic pathways whereby lecithin, and other phosphatides, are formed. These pathways were worked out using, for the most part, enzymes derived from liver tissue. In comparison to chicken liver studied by Kennedy (2, 4), adult brain and peripheral nerve are rather poor sources of the enzymes concerned with the biosynthesis of glycerophosphatides. However, using synthetic intermediates labeled with radioactive isotopes, it has been possible to show that brain and peripheral nerve contain

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[2] Studies carried out during the tenure of a Lederle Medical Faculty Award.

enzymes capable of catalyzing all of the key steps in the biosynthesis of lecithin.

Figure 2 illustrates an overall scheme for the P^{32}-labeling of lecithin. It is based largely on the work of Kennedy (2, 4) for liver, and is supported by our work on rat brain (5). Enzymes responsible for the transfer of phosphate from ATP to choline, with the formation of phosphorylcholine (PCh), and to glycerol, with the formation of L-α-glycerophosphate (L-α-GP), have been described by Wittenberg and Kornberg (6) and Bublitz and Kennedy (7), respectively. Another route for the formation of L-α-GP may be by way of the glycolysis intermediate, dihydroxyacetone phosphate. An active L-α-GP dehydrogenase is known to be present in brain (8).

FIG. 1. Labeling of glycerophosphatide.

In table 1, *experiment 1*, taken from the results of McMurray, Strickland, Berry and Rossiter (5), shows that the P^{32} of α-GP^{32} may be incorporated into the phosphatide of either phosphorylating mitochondria or glycolysing water homogenates of rat brain. Examination of the labeled phosphatide by the method of Dawson (9) indicated that the product of the reaction was phosphatidic acid-P^{32}. The labeling, which was

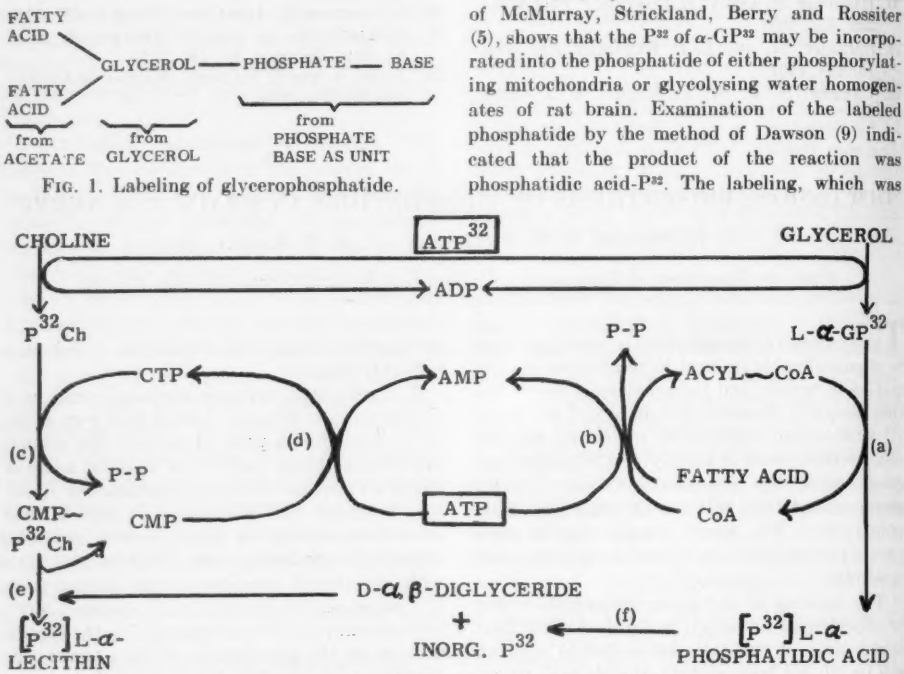

FIG. 2. Scheme representing P^{32}-labeling of lecithin in brain preparations.

TABLE 1. IN VITRO LABELING OF PHOSPHATIDES IN RAT BRAIN AND SCIATIC NERVE
(c.p.m./μg. P)

Exp. No.	Precursor	Additions	Mitochondria (gas phase, O_2)	Water Homog. (gas phase, N_2)
1	α-GP^{32}	Control	25.4	13.4
		CoA (5×10^{-5} M)	89.2	35.5
2	$P^{32}Ch$	Control	1.7	1.7
		CTP (3.5×10^{-4} M)	31.7	32.9
3	CMP-$P^{32}Ch$	Control	48.6	35.0
		D-α,β-diglyceride	81.0	54.8
		Fraction Labeled	Water Homog. (Control)	Water Homog. (D-α,β-diglyceride)
4	CMP-$P^{32}Ch$	Total lipid P	34.1	43.9
		Lecithin	77.3	102
		Phosphatidyl ethanolamine	2.7	2.1
		Phosphatidyl serine	1.0	2.2
		'Sphingomyelin'	6.5	
			Sciatic Nerve Homog. (Control)	Sciatic Nerve Homog. (degen. 13 days)
5	CMP-$P^{32}Ch$	Total lipid P	2.4	12.7

by way of *reactions a* and *b* (fig. 2), first described by Kornberg and Pricer (10, 11) for liver tissue, was greatly stimulated by the addition of coenzyme A (CoA). Moreover, metabolic energy was required for the labeling of the phosphatidic acid, since the labeling was abolished by the addition of inhibitors that prevented the production of ATP, or by the omission of ATP from the reaction mixture.

Experiment 2 (table 1) shows that the P^{32} of $P^{32}Ch$ also may be incorporated into the phosphatide of both rat brain preparations. In this instance, however, examination of the labeled phosphatide revealed that the greater part of the radioactivity was in the lecithin. It would seem that the labeling occurred by way of *reactions c* and *d* (fig. 2), involving the novel intermediate cytidine diphosphate choline (CMP-P^{32}Ch) described by Kennedy and Weiss (2), since the reaction was dependent upon the presence of cytidine triphosphate (CTP). None of the other nucleoside triphosphates tested could replace CTP, and the addition of CoA was without effect. Again metabolic energy was necessary for the labeling.

Experiment 3 shows that synthetic CMP-P^{32}Ch was incorporated into the phosphatide fraction of both rat brain preparations and that the incorporation was stimulated by the addition of D-α,β-diglyceride, according to *reaction e* (fig. 2). In this experiment the diglyceride was prepared enzymatically from purified egg lecithin by the method of Hanahan and Vercamer (12), using the lecithinase D of *Clostridium perfringens*[3]. Weiss, Smith and Kennedy (13) suggested that D-α,β-diglyceride may be formed from phosphatidic acid by *reaction f* (fig. 2). It will be noted that the P^{32} of the labeled lecithin is derived from $P^{32}Ch$ by way of CMP-P^{32}Ch. The label of phosphatidic acid-P^{32} is lost as inorganic P^{32} by *reaction f*, and does not enter the lecithin molecule.

Experiment 4 (table 1) shows the results of a similar experiment with D-α,β-diglyceride prepared enzymatically from lecithin isolated from spinal cord by the method of Pangborn (14). The difficulty arising from the relative insolubility of diglycerides has been commented upon by Kennedy and Weiss (2). In this experiment the spinal cord diglyceride was emulsified in serum albumin (0.02%), whereas in *Experiment 3* the egg diglyceride was emulsified in 'Tween 20' (0.025%).

Separation of the hydrolysis products of the labeled phosphatide (*experiment 4*) showed that, in comparison to lecithin, there was negligible labeling of the other glycerophatides. The fraction designated 'sphingomyelin' refers to the portion of the phosphatide fraction that was stable to the Schmidt-Thannhauser (15) alkaline hydrolysis procedure. The presence in this fraction of phosphosphingolipides that do not contain choline would necessitate the revision of the specific activity of sphingomyelin in an upward direction.

Experiment 5 (table 1) shows that the phosphatide fraction of an homogenate of rat peripheral nerve also was labeled from CMP-P^{32}Ch and that the labeling was greatly increased during Wallerian degeneration.

CONCLUSIONS

Experiments on the *in vitro* labeling of glycerophosphatides from a variety of precursors provide evidence that in brain and peripheral nerve these phosphatides are formed *in situ* from small molecules.

The pathway for the biosynthesis of lecithin in brain and peripheral nerve is similar to that described by Kennedy (2, 4) for chicken liver.

When the population of Schwann cells in a peripheral nerve is increased by sectioning the nerve, the labeling of the phosphatides is increased. It is suggested that this represents an increase in the potentiality of the nerve for remyelination.

[3] Kindly supplied by Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

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AMERICAN ASSOCIATION OF IMMUNOLOGISTS

Symposium on Non-Specific Effects of Gram-Negative Bacteria and Polysaccharide Complexes on Resistance to Infection and on Antibody Formation[1]

CHAIRMAN: MERRILL W. CHASE

EFFECTS OF BACTERIAL ENDOTOXINS ON SUSCEPTIBILITY TO INFECTION WITH GRAM-POSITIVE AND ACID-FAST BACTERIA

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INJECTION into experimental animals of sublethal doses of bacterial endotoxins (lipopolysaccharides) is known to first depress, then to increase resistance to infection with gram-negative bacilli unrelated to the strain from which the endotoxin was prepared. We have found that this biphasic, non-specific effect on resistance applies also to infection with gram-positive and acid-fast organisms, and that it can be elicited by injection of cellular constituents of tubercle bacilli and of pertussis vaccine.

Most of our experiments were carried out in albino mice raised at the Rockefeller Institute, ranging from 4 to 8 weeks of age. The materials under investigation were injected by the intraperitoneal route and the infective agents by the intravenous route. Young cultures of either one of three species possessing high virulence for mice, *Staphylococcus pyogenes*, *Mycobacterium fortuitum*, *Mycobacterium tuberculosis*, were used for the challenge infections.

Injection into mice of doses ranging from 5–50μg of lipopolysaccharide caused a rapid but transient loss of weight (approximately 10%) from which the animal recovered completely in 2–3 days. Animals so treated consistently exhibited a much enhanced resistance to staphylococci and to mycobacteria when the challenge infective dose was injected 1 week to 1 month later. In several experiments, indeed, the protective effect has been found to last for several months.

In contrast, resistance to infection was much depressed when the dose of lipopolysaccharide was administered within a very few hours either before or after, or simultaneously with, the challenge infection. The depressing effect on resistance could be brought out even more dramatically by administering the lipopolysaccharide to animals infected months previously with sublethal doses of staphylococci or mycobacteria. By this technique, the chronic infection was converted into an acute process, the explosive multiplication of the bacteria often resulting in death of the animal.

The results outlined above have been obtained with several preparations of lipopolysaccharides derived from various species of gram-negative bacilli and obtained through the courtesy of Dr. Maurice Landy (National Institutes of Health), Dr. H. W. Schoenlein (Difco Laboratories), and Dr. Otto Westphal (Dr. A. Wander Forschungsinstitut, Germany). More recently we have found that a biphasic, non-specific effect on resistance can be elicited also by injecting either pertussis vaccine, or killed tubercle bacilli, or extracts of

[1] Chicago, Illinois, April 17, 1957.

these organisms. Treatment of mice with 1 mg of material derived from cultures of BCG or H37RV or with 0.05 ml of pertussis vaccine (containing approximately 60 billion cells) was found to convert long-standing chronic infections with staphylococcus or mycobacteria into acute infectious processes. In contrast, administration of the same doses (or less) of these bacterial products to uninfected animals resulted in an increase in their resistance to subsequent infection with various types of pathogens. This increase in resistance lasted for several months.

HOST RESPONSES ELICITED BY POLYSACCHARIDES OF BACTERIAL AND MAMMALIAN DERIVATION

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IT HAS long been known that certain bacterial products may alter non-specific defense mechanisms of the host, but it is only recently that their effects on resistance have been studied in any detail. The administration of viable or killed gram-negative organisms, or their cell walls, rapidly gives rise in mice to a transient, non-specific resistance to infection with gram-negative pathogens (1). This increase in resistance frequently is preceded by a decrease, i.e., a negative phase. Pillemer *et al.* found that such gram-negative pathogens, or their cell walls, interact with the properdin system (2).

This system, which comprises properdin, complement and magnesium ions, exerts a marked bactericidal effect on gram-negative bacteria (3). These observations suggested that a kind of component known to be common to gram-negative organisms, (variously identified as somatic antigen, lipopolysaccharide, or endotoxin) might be the material in these bacteria responsible for the foregoing effects. That is to say, the cell surface containing this type of complex polysaccharide may be the site of reaction with properdin, and perhaps with other host defensive agents involved in non-specific resistance. On this basis it was anticipated that, in the isolated state, these polysaccharides not only would be active in modifying host resistance, but would be considerably more active than the organisms from which they were obtained. This has indeed proved to be the case, for it was found that polysaccharide endotoxins from various gram-negative bacteria were highly effective in altering serum properdin levels of mice (4) and their non-specific resistance to gram-negative infections (5).

Many factors were shown to influence these two effects which follow the administration of bacterial endotoxins in mice. Four primary variables—*a*) nature of the polysaccharide preparation, *b*) dose, *c*) interval between polysaccharide administration and challenge, and *d*) challenge agent—were studied in some detail. For any given polysaccharide and any gram-negative challenge organism the critical relationships were brought out in a series of dose-time experiments. These generally encompassed the effect on resistance and on serum properdin levels in mice which had received a bacterial polysaccharide in amounts ranging from 0.1 to 100 μg administered at intervals varying from 120 hours to 1 hour prior to challenge. High dosage generally resulted in an initial depression of resistance, which was followed by an increase that persisted for 5 days or more. Low dosage produced only a swift rise in resistance, which was of progressively shorter duration as the dose of polysaccharide was decreased. Alterations in serum properdin levels followed a similar pattern, i.e., a large amount of polysaccharide caused marked depression in properdin titer followed by a sustained elevation, while a lesser amount caused only an elevation. These experiments indicated that the rate at which these two host responses developed, their magnitude and their duration were functions of the dose of polysaccharide and the timing of its administration. Analogous effects on resistance were observed in mice challenged with the following gram-negative pathogens: *Salmonella typhosa*, *Escherichia coli*, *Proteus vulgaris*,

Pseudomonas Aeruginosa and *Klebsiella pneumoniae*. The changes in resistance and the effects on serum properdin were elicited with 26 polysaccharide endotoxins isolated from gram-negative bacterial species; 6 genera, numerous serotypes, and colonially smooth and rough strains were represented. These products had been prepared in six different laboratories in which widely differing isolation techniques were employed. It is thus evident that this type of complex polysaccharide, capable of altering resistance to infection and serum properdin levels in mice, is common to gram-negative bacteria.

The foregoing summarizes, in part, the status of the problem as developed by investigators in other laboratories and as described in two recent publications by Landy and Pillemer (4, 5). The rest of this talk summarizes more recent work (6, 7) which is now in press. It is not possible this evening to furnish the details of the experiments but only the main ideas together with some illustrative data.

Bacteria have been considered to be the only source in nature of substances with the properties of endotoxins, including the two attributes just described. So far as we are aware, materials capable of evoking this characteristic spectrum of host effects have not, until now, been recognized in other natural sources. Recent work in this laboratory has indicated that certain polysaccharides derived from animal and plant tissues elicit this same array of responses. The concept and observations which led to this work are as follows: polysaccharides from gram-negative bacteria induce hemorrhage and necrosis in sarcomas in animals and man, as attested by the studies of Shear and associates over a period of many years. Shear was impressed by the fact that tumor tissue responded to a single dose of polysaccharide without the necessity for a preparatory dose, as is required in normal tissue, for example, in the Shwartzman reaction. It was therefore postulated by Shear (8) that tumors might already be 'prepared' in consequence of their containing a component analogous to the polysaccharides of gram-negative bacteria. On the basis of this concept, tumors were fractionated in a search for polysaccharide components similar to those in such bacteria. The isolation procedures used were those previously employed with bacteria, and polysaccharides were isolated which damaged tumors, were pyrogenic, and Shwartzman-active (9). Tissues from normal animals and plants (10) were then examined and, here too, many were found to contain 'active' polysaccharides. Details of their preparation, and data on their physical and chemical properties, are being reported separately.

TABLE 1. POLYSACCHARIDE PREPARATIONS STUDIED

Source	Min. Pyrogenic Dose, μg	Source	Min Pyrogenic Dose, μg
Mouse kidney	0.3	Rabbit skin	0.5
Mouse lung	0.2	Chick embryo skin	41
Mouse liver	0.2	Tangerine	40
Mouse stomach	0.3	Bryonia root	1
Mouse sarcoma	0.02	Serratia marcescens	0.007
Mouse carcinoma	0.1	Salmonella typhosa	0.001

The number of host polysaccharides of varied origin, isolated in this laboratory from animal and plant sources, was so large as to make impractical a detailed examination of the biological properties of all. Consequently, from more than 100 specimens, 10 were selected for systematic study, primarily for comparison of their 'endotoxic' properties with those of two typical endotoxic polysaccharides from bacteria. Selection was made on the following basis: *a*) tumor-damaging potency; *b*) tissue source, viz., plant or animal, and normal or neoplastic. The sources of these tissue polysaccharides are given in table 1, together with their activity in one illustrative host response, i.e., their pyrogenic activity in rabbits.

Twelve characteristic host effects produced by endotoxins were studied with this group of tissue polysaccharides. These effects are listed in table 2, which also illustrates, for one of these polysaccharides, potency in eliciting these reactions. These two protocols summarize the criteria of endotoxic activity applied to the study of a selected group of polysaccharides of animal, plant and bacterial derivation. The performance of these products is scored in table 3, where the numerator of the fractions represent the number of host effects elicited; the denominators give the total number of reactions examined with each. Three of the preparations were consistently negative except for some pyrogenic activity; the other tissue polysaccharides evoked the entire array of host reactions typically produced by bacterial endotoxins, including alterations in non-specific resistance and interaction with the properdin system. In most of these reactions, larger amounts of the polysaccharides from

TABLE 2. HOST RESPONSES TO TISSUE POLYSACCHARIDES*

Response	Amt. Required, μg	Response	Amt. Required μg
Fever	0.3	Dermal hemorrhage and necrosis by adrenaline	<50
Fever tolerance	<20	Enhancement of antibody production	<100
Leucocytic changes	<10	Lethality for rabbits	<250
Shwartsman (preparatory)	100	Alteration of resistance to infection	4
Shwartsman (provocative)	300	Alteration of serum properdin levels	5
Tumor damage	70	Appearance of high mol. wt. material in serum	5

* E. g. mouse kidney polysaccharide.

animal and plant sources were required than of those from gram-negative organisms; nevertheless, the qualitative similarity in their endotoxic properties was striking.

To those who have long been accustomed to associate these reactions only with materials of microbial origin, these findings might be disconcerting at first. However, similarities in the biochemistry of bacteria and of cells from structurally more complex organisms have become increasingly evident as knowledge has increased. In retrospect it is consistent that mammalian tissues should yield substances such as these, with properties so similar to those previously recognized only in bacteria. It might appear strange that mammalian organs should be such a relatively rich source of substances which are capable of producing marked physiological disturbances in the very species from which they derived. However, preliminary evidence indicates that their endotoxic activities are manifest only when they are released from their native state of firm combination with other cellular components. Much remains to be learned about the origin, distribution, and state of combination of these host polysaccharides, and about factors which influence their interactions.

Of course it is too early to estimate the significance of these findings. Nevertheless, it is of interest to consider a few of the many possible implications. The demonstration that mouse tissue polysaccharides cause marked changes in the defensive capabilities of this species provides a basis for fresh consideration of some aspects of natural resistance. For example, the marked changes in resistance, properdin levels, and activity of the reticuloendothelial system, which are known to follow the administration of exceedingly small amounts of bacterial polysaccharides, may involve the release and participation of tissue polysaccharides. Moreover, in the profound toxemia frequently associated with enteric fevers in man, the absence of large numbers of bacteria in the circulation has been puzzling; this anomaly might be accounted for by postulating the release of such tissue polysaccharides.

Some of the physiological disturbances elicited by relatively large amounts of tissue polysaccharide may well be injurious to the host. On the other hand their liberation in small amounts may be advantageous, i.e., in maintenance of properdin levels and in the stimulation of other defensive mechanisms. This could constitute a contribution toward the maintenance of natural resistance.

There are many kinds of injuries which, when of sufficient severity, evoke in the host a syndrome with some features in common with that produced by bacterial endotoxins. The possibility has long been considered that toxic substances appear in tissue areas subjected to severe trauma, or to anoxia associated with hemorrhage or circulatory occlusion. Such postulated substances have been viewed as contributing to the irreversibility of the shock syndrome. The impressive studies of Fine (11) and his associates have shown that hemorrhagic shock of even brief duration leads to peripheral vascular collapse with an impairment of the antibacterial defenses. This was attributed to bacteria ordinarily present. However, the presumptive offending bacteria were not demonstrable in the blood or tissues of animals in shock. Our finding in mammalian tissues of substances with the biological properties of endotoxins suggests an additional or alternative explanation.

It is suggested that tissues damaged in any one of many ways release into the circulation endogenous polysaccharides in a form and in an

TABLE 3. NUMBER OF HOST RESPONSES TO EACH POLYSACCHARIDE

Source	Responses Elicited/ Tested	Source	Responses Elicited/ Tested
Mouse kidney	12/12	Rabbit skin	1/10
Mouse lung	10/10	Chick embryo skin	1/10
Mouse liver	9/10	Tangerine	1/10
Mouse stomach	10/10	Bryonia root	10/12
Mouse sarcoma	12/12	Serratia marcescens	10/10
Mouse carcinoma	10/12	Salmonella typhosa	12/12

amount which produce the series of physiological disturbances which characteristically follow severe injury. It is thus visualized that the reaction of the host in some infections, and in other pathologic states, may be greatly influenced by substances such as these polysaccharides which are present in its own tissues.

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EFFECT OF BACTERIAL ENDOTOXINS ON THE RETICULOENDOTHELIAL SYSTEM[1]

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The large molecular weights and colloidal characteristics of bacterial endotoxins make these substances readily phagocytized by the reticuloendothelial system.

The work of Beeson and Bennett (1, 2) provided the first evidence showing the importance of the reticuloendothelial cells in the reaction to bacterial endotoxins. These authors reported that induced tolerance to the endotoxin was associated with a more rapid clearance of the lipopolysaccharide from the blood by the reticuloendothelial system. Intravenous administration of large amounts of colloids with the purpose of interfering with the normal function of the reticuloendothelial cells caused the tolerant rabbits to lose their tolerance.

Thomas and Good (3, 4), investigating this problem further in rabbits, confirmed and extended the earlier findings of Beeson and Bennett. They showed the lethal effect of endotoxins to be considerably enhanced by attempts to block the reticuloendothelial system with a variety of colloidal agents. By these means, prostration and death could be produced with doses of endotoxin smaller than one thousandth of the amount required to cause symptoms in normal animals. Furthermore, the typical lesions of the generalized Shwartzman reaction were produced in a high proportion of animals with one injection of endotoxin if they had been previously injected with a large dose of a colloidal suspension.

These observations, together with the availability of techniques developed recently to investigate quantitatively the phagocytic function of the reticulo-endothelial system (5–8), led us to study the effects of gram-negative bacteria and their endotoxins on the reticuloendothelial system in mice (9) and rabbits. We were able to show that gram-negative bacteria and their purified lipopolysaccharide have considerable effects on the reticuloendothelial cells, which can be summarized as follows: within a few hours after an intravenous or intraperitoneal injection of these agents in suitable amounts, the phagocytic cells of the liver and spleen are found to be injured and show a deficiency in clearing the blood of injected colloids; their ability to metabolize phagocytized material is also impaired. These effects are dose-dependent, and are followed after recovery by a period of increased phagocytic activity of the reticuloendothelial system, which may be due to new cell formation. A previously

[1] This work has been supported by a Public Health Service Rheumatic Pathogenesis grant.

administered small dose of endotoxin conditions the reticuloendothelial system in such a way that larger doses administered later no longer cause injury to the reticuloendothelial cells, but rather have an immediate stimulating action on the reticuloendothelial system. These data are in full agreement with the results of Landy and Pillemer (10) on the effect of small doses of endotoxins on resistance to infections; for the infective agent often can be likened to a challenge with a larger dose of an endotoxin.

METHODS

Ever since the term 'reticuloendothelial system' was introduced by Aschoff to describe the cells capable of extracting from their environment and concentrating in their cytoplasm dyes and colloidal particles, its naming, its function and the numerous experiments dealing with its blockade have stimulated considerable criticism. We feel bound, therefore, to define for the sake of clarity specifically what we are measuring. We have considered under the generic name of reticuloendothelial system the macrophages lining the sinusoids of liver, spleen, lymph nodes, bone marrow, monocytes of the blood and histiocytes of connective tissues. Let us stress, however, that only the macrophages in contact with the circulating blood lend themselves to quantitative investigation with the available methods, and our findings apply exclusively to them. Their phagocytic function is studied by measuring the clearance from the blood of suitable colloidal suspensions which do not cross the capillary wall and which are selectively and efficiently phagocytized by these cells (5, 8). When proper doses of colloids are injected, the rate of clearance is a measure of the phagocytic activity of these macrophages. The distribution of the phagocytized colloids among the various organs is also investigated. When suitable colloidal particles are injected intravenously, almost all of the injected material is picked up by the macrophages of the liver and spleen (about 90%). When very small doses of colloids are injected, the liver is able to clear nearly all the injected particles (8, 11); because of its predominant blood supply, it acts as the principal filter for particulate matter in the blood.

The injected colloidal particles are cleared from the blood according to an exponential function of time:

$$(\text{Log } C_1 - \text{Log } C_2)/(T_1 - T_2) = K, \quad (1)$$

where C_1 and C_2 are the concentrations of carbon measured respectively at times T_1 and T_2. However, what characterizes blood clearance of colloids by the reticuloendothelial system is the fact that, above a critical dose of injected colloids, the rate of clearance, K, varies inversely with the dose, while still remaining a first order rate:

$$K \times D = \text{constant} \quad (2)$$

The constant K of *equation 1* has been called the phagocytic index and is a measure of phagocytic activity for a given dose of a colloid above the critical dose.

When very small quantities of colloid (below a critical dose) are injected the clearance is very rapid and independent of the dose; practically all of the injected colloid is found in the liver. The phagocytic capacity of the cells investigated is in excess of the amount of particles injected. In this range of dosage, the colloid clearance test in the normal animal is only a measure of liver blood flow (8, 11). In this range again, the injected colloid, be it albumin complex or chromium phosphate or carbon, is removed very efficiently by the Kupffer cells, so that 80–96% of the particles in the blood, depending upon the colloid, are picked up in one passage. We call this 'maximum efficiency of colloid clearance.' As we shall see, when the cells are injured by endotoxin, their maximum efficiency of clearance decreases considerably, although the blood flow through the organ remains unchanged.

Above a critical dose of colloid, the particles are in excess of cellular capacity. This allows an efficient assay of the phagocytic activity of the reticuloendothelial cells. The rate of clearance is then directly dose-dependent. In this range, K is a measure of phagocytic activity of the cells, if blood flow is normal.

The critical dose of colloid, at which the rate of clearance becomes dose-dependent, is a specific measure of the phagocytic capacity of the reticuloendothelial system, independently of blood flow. It can be easily measured by investigating clearance rates in the two dose ranges and computing it from *equation 3*:

Critical R.-E. dose

$$= (K \times D)/\text{Max. value of } K \quad (3)$$

Therefore, in order to evaluate the behaviour of the reticuloendothelial system towards injected colloids, we can use several criteria:

1) The rate of clearance, K, for a suitable dose

of colloid and the index α corrected for relative variations of the size of the liver and spleen from *equation 4* (5, 8)

$$\alpha = (W/Wls)\sqrt{K} \qquad (4)$$

where W refers to body weight and Wls to combined weights of liver and spleen.

2) The amount of liver blood flow per unit of time.

3) The maximum efficiency with which colloids are removed by the Kupffer cells of the liver when present in minute concentration.

4) The critical reticuloendothelial dose, which is a specific measure of phagocytic capacity of the reticuloendothelial cells.

In the experiments which are reported here, 3 different colloids were used:

1) Carbon particles suspended in gelatin, very homogeneous in size, measuring about 250 Å, (preparation cll 1431/a, secured from Gunther Wagner, Hanover, Germany); *2*) Chromium phosphate labeled with P^{32} prepared by Dr. Dobson (10); and *3*) Heat denatured human serum albumin labeled with I^{131} (8). Iodine-labeled denatured proteins are readily phagocytized by the reticuloendothelial system (12, 13) and are very rapidly broken down by the cells. The iodine is released from the macrophages. The study of this phenomenon provides a measure of the metabolic function of the reticuloendothelial cells.

EFFECT OF BACTERIAL ENDOTOXIN ON PHAGOCYTIC FUNCTION OF RETICULOENDOTHELIAL SYSTEM IN RABBITS

Since the rabbit is the animal species most susceptible to the damaging effect of endotoxin (14), the latter was investigated particularly in young rabbits weighing 1.2–1.5 kg. They were injected intravenously with 20 γ of purified lipopolysaccharide from *Salmonella abortus equi* A.E. 1131S, prepared by Dr. Otto Westphal. This dose, although occasionally lethal to some rabbits, was generally well tolerated. At various time-intervals afterwards, the phagocytic activity of the reticuloendothelial system was investigated in groups of rabbits by injecting 4 mg. C/100 gm body weight and measuring the rate of clearance from the blood as expressed by the phagocytic index K and the corrected index α which measures tissue activity. The results obtained are presented in figure 1, together with the values of K and α and their standard deviations obtained in control rabbits injected with the same dose of carbon, and recorded at 0 time.

After 72 hours, the same dose of endotoxin was repeated. The results, presented in figure 1, show that the rate of clearance of injected carbon is significantly decreased after a single injection of a moderate dose of endotoxin. This effect is maximal at 12 hours and is still observed 24 hours after endotoxin. However, after 48 hours the rabbits show a slightly increased rate of carbon clearance, as both K and α are found to be outside the upper limit of the normal standard deviation. A second identical dose of endotoxin administered at 72 hours no longer causes a detectable effect, as the phagocytes seem to be able to handle the endotoxin differently when challenged again at a suitable time.

In order to determine whether the decreased rate of clearance of carbon observed maximally at 12 hours was due to a damaging effect of the endotoxin on the cells, measurements of maximum efficiency of clearance of chromium phosphate and albumin complex by the liver Kupffer cells and measurements of liver blood flow were carried out in some rabbits and compared with normal values.

The techniques used involved the injection of minute amounts of these two efficiently phagocytized colloids in the inferior mesenteric veins, so that they be filtered by the Kupffer cells of the liver before reaching the general circulation. The maximum efficiency of clearance and liver blood flow were computed as described (8, 11). The results of these experiments are presented in table 1. They show that the values for liver blood flow are of the same order of magnitude in the control animal and in rabbits 12 hours after 20 γ of endotoxin and are comparable with the published values for normal rabbits (8, 11). The ability of the Kupffer cells to remove colloids from the blood is markedly decreased, even when present in very low concentrations. The maximum efficiency of clearance of chromium phosphate is decreased from over 90% to 76% and 49% in two cases, and the efficiency of clearance of albumin complex drops from the normal value of about 80% to 45% and 14% in two cases.

These findings are in full agreement with the histological appearance of the liver, spleen, lung and kidneys. The macrophages of the spleen and liver of the endotoxin-treated rabbit are not able to pick up normally and concentrate in their cytoplasm the injected colloids (such as carbon), while the vascular endothelia of other organs,

such as lungs, kidney glomeruli, etc., show signs of injury as revealed by abnormal deposition of carbon.

In the course of our studies on the phagocytic activity of the reticuloendothelial system, we have observed that the presence in the blood of any colloids susceptible to phagocytosis by the macrophage system caused a competitive inhibition of phagocytosis of other colloids such as carbon particles (7, 12, 13, 15). This could be demonstrated in mice, for instance, by injecting during the course of carbon clearance a competing colloid, such as heat denatured serum protein, albumin complex or saccharated iron oxide.

The blood of rabbits whose reticuloendothelial system has been damaged by endotoxin was found to contain large amounts of phagocytizable colloids of undetermined origin. Two independent observations led us to entertain this hypothesis: *1*) The sera of these rabbits were generally unusually cloudy and opalescent, and *2*) Thomas *et al.* reported earlier the presence in the plasma of endotoxin-treated rabbits of a denatured form of fibrinogen precipitable by heparin in the cold (16). The plasma and serum of endotoxin-treated rabbits were investigated for the presence of material susceptible of being phagocytized by injecting 0.2 ml/20 gm intravenously into mice during the course of their clearance from the blood of 16 mg C/100 gm body weight. The clearance rate of carbon was measured before and after the injection of the plasma or serum to be assayed. The same experiments were performed with plasma or serum from control rabbits. The results of these experiments are presented in figures 2 and 3; they show that both plasma and serum of rabbits treated with endotoxin interfere with carbon clearance presumably by competition, owing to some phagocytizable substances present. This effect is a little more marked with plasma than with serum. Control plasma or serum from normal rabbits showed no action. The nature of these substances has not been determined and may possibly be modified fibrinogen. It is possible that the damaging effect caused by endotoxin on the reticuloendothelial cells prevented the normal removal of these

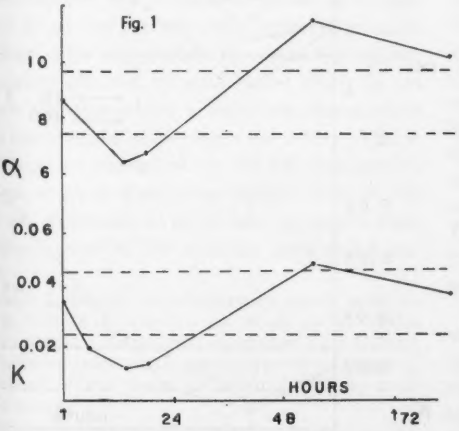

FIG. 1. Effect of endotoxin on phagocytic activity of reticuloendothelial system in rabbits measured by phagocytic indices K and α for the dose of 4 mg C/100 gm. *Arrows* indicate injection of 20 γ *Salmonella abortus equi* endotoxin. *Broken lines* are limits of standard deviation in control rabbits.

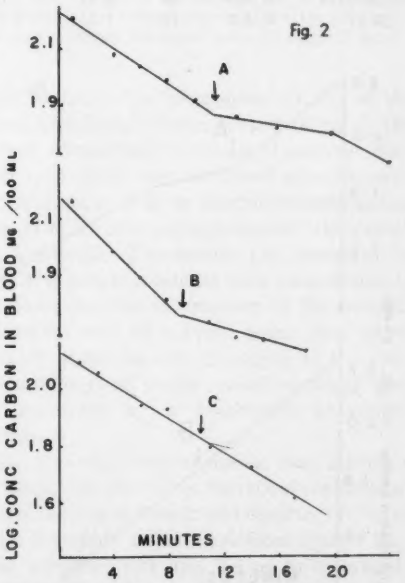

FIG. 2. Effect of plasma from rabbit treated with endotoxin on phagocytosis of carbon particles by the reticuloendothelial system in mice. Dose of carbon, 16 mg/100 gm. *A* and *B*, 0.2 ml plasma from rabbit injected with 20 γ *Salmonella abortus equi* endotoxin. *C*, 0.2 ml plasma from control rabbit.

substances from the circulation and caused their abnormal accumulation in the blood.

EFFECT OF BACTERIAL ENDOTOXIN AND OF ZYMOSAN ON RETICULOENDOTHELIAL SYSTEM IN MICE

The damaging effect of endotoxins on the macrophages was also investigated in mice, as well as the hyperactive phase that follows; the latter can be demonstrated much more easily in mice (9) than in rabbits. We have investigated the effect of 10 γ and 100 γ of *E. coli* 0.8 lipopolysaccharide (prepared by Dr. Landy) on the phagocytic activity of the reticuloendothelial cells. These experiments were performed at Dr. Landy's suggestion, with the doses and the preparation that have been shown by Landy and Pillemer to protect mice against infection and to modify serum properdin levels. At various time intervals after the intravenous or intraperitoneal injection of the endotoxin, the rates of clearance of 16 mg C/100 gm body weight were investigated, and the indices K and α were calculated from *equations 1* and *4*. These results are presented in figure 4, together with the mean values of K and α with their respective standard deviations obtained in 25 control mice and presented at 0 time.

TABLE 1. EFFECT OF BACTERIAL ENDOTOXINS ON EFFICIENCY OF COLLOID CLEARANCE BY KUPFFER CELLS OF LIVER AND ON LIVER BLOOD FLOW IN THE RABBIT, MEASURED 12 HR. AFTER I.V. INJECTION OF 20 γ SALMONELLA ABORTUS EQUI ENDOTOXIN

	Ke	Efficiency, %	Min. Liver Blood Flow, ml/gm/min.	Corr. Liver Blood Flow, ml/gm/min.,
20 γ human albumin complex labeled with I^{131}, C.A.I.[131]				
Control	0.644	82	1.23	1.5
Endotoxin, 20 γ	0.345	45	0.585	1.3
	0.138	14	0.21	1.5
Chromium phosphate labeled with P^{32}				
Control	0.552	90	1.15	1.28
	0.690	94	1.6	1.7
Endotoxin, 20 γ	0.299	49	0.51	1.04
	0.575	76	1.3	1.7

Both 10 γ and 100 γ of *E. coli* endotoxin cause a transitory depression of the phagocytic activity of the reticuloendothelial system. This action is more severe and longer lasting with 100 γ than with 10 γ. Twenty four hours after 10 γ, this effect can no longer be observed. The same results were obtained whether the endotoxin was in

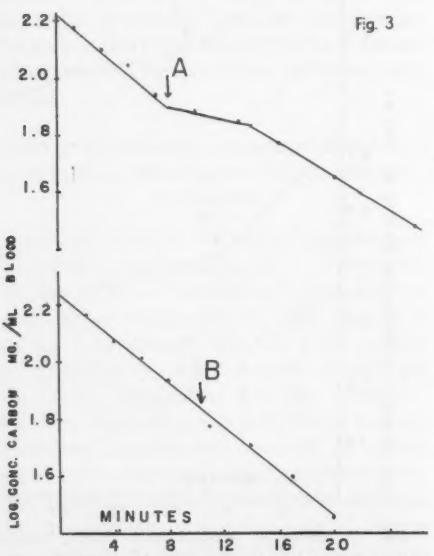

FIG. 3. Effect of serum from rabbit treated with endotoxin on phagocytosis of carbon particles by the reticuloendothelial system in mice. Dose of carbon, 16 mg/100 gm. *A*, 0.2 ml serum from rabbit injected with 20 γ *Salmonella abortus equi* endotoxin. *B*, 0.2 ml of serum from control rabbit.

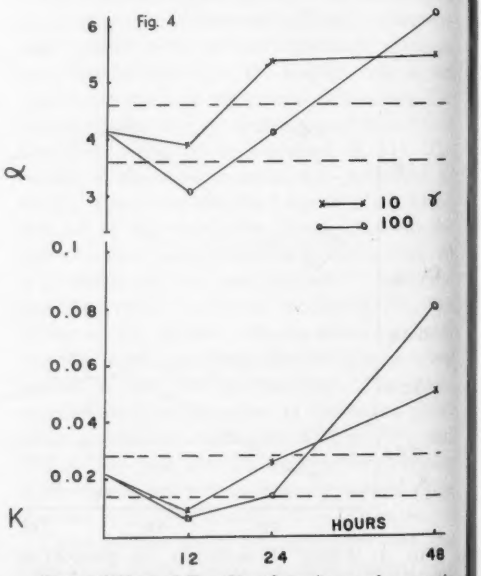

FIG. 4. Effect of *E. coli* endotoxin on phagocytic function of reticuloendothelial system in mice measured by the indices K and α for the dose of 16 mg C/100 gm, 10 γ and 100 γ of *E. coli* endotoxin being injected at 0 time. *Broken lines* indicate limit of normal standard deviations.

jected intravenously or intraperitoneally. The period of depression was followed—within 24-hours for 10 γ and 48 hours for 100 γ of endotoxin—by a very considerable stimulation of the phagocytic function of the macrophage system. It should be stressed that the period of depression of the phagocytic activity follows fairly closely for both 10 γ and 100 γ the increased susceptibility to infection with gram-negative organisms observed by Landy and Pillemer (10), while the second phase of reticuloendothelial hyperactivity is observed at the time when mice are more resistant to infection with gram-negative bacteria and show increased serum properdin levels (10).

The effect of a toxic dose of the lipopolysaccharide on the ability of the cells to break down phagocytized material was also studied. Human albumin complex labeled with I^{131} was used in experiments carried out with the technique described (8). It was observed that, within a few hours after the injection of a toxic dose of *E. coli* lipopolysaccharide, the ability of the liver Kupffer cells to break down phagocytized albumin complex and to release radioactive iodine is severely impaired, depending upon the dose of endotoxin used. We have observed, both with Dr. Landy's preparation and other preparations (9), that a previous treatment with a small dose of lipopolysaccharide conditions the mice so that a larger dose, which normally would cause a depression of the reticuloendothelial cells, does not do so but renders them hyperactive much sooner. This may explain the protection against infection afforded by small doses which do not show, by themselves, a highly significant effect on reticuloendothelial phagocytic activity. These findings are illustrated by the following experiment. A group of mice was injected with 10 γ of *E. coli* endotoxin; 24 hours later, several of these animals received 100 γ of the same endotoxin.

TABLE 2. EFFECT OF A PREVIOUS SMALL DOSE OF BACTERIAL ENDOTOXIN (E. COLI) ON RESPONSE OF RETICULOENDOTHELIAL SYSTEM TO A LARGER DOSE OF SAME TOXIN, EXPRESSED BY PHAGOCYTIC INDEX K FOR DOSE OF 16 MG C/100 GM BODY WEIGHT

	Values of K		
25 Control mice (C)	10 γ, followed 24 hr. later by 100 γ, endotoxin. C 24 hr. after last injection	100 γ endotoxin 24 hr. before C	10 γ endotoxin 48 hr. before C
0.021 ± 0.007	0.099	0.014	0.051

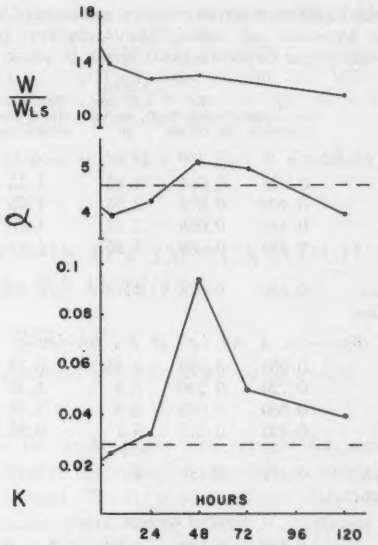

FIG. 5. Effect of 1 mg zymosan i.v. on phagocytic function of reticuloendothelial system in mice measured by the indices K and α for the dose of 16 mg C/100 gm. *Broken lines* indicate upper limits of normal standard deviations for K and α respectively. The values shown at 0 time represent the mean values obtained with 25 control mice.

After 24 hours, the phagocytic activity of the reticuloendothelial system toward 16 mg C/100 gm was investigated. The mean phagocytic index K of each group was calculated and compared with the value of K in control animals and in mice 24 hours after a single dose of 100 γ of *E. coli* endotoxin. The results are presented in table 2. A previous contact with a small dose of endotoxin modifies the reaction of the reticuloendothelial cells to a much larger dose which normally would be very damaging to the cells. The cells respond to the second challenge with the endotoxin by a heightened phagocytic activity.

The fact that several agents that stimulate the phagocytic activity of the reticulo-endothelial system, such as endotoxin and dextran, (17) have also a favorable action on defense against bacterial infections (10, 18), led us to investigate the effect of zymosan, which has been reported by Kiser and associates (18) to protect against experimental infection.

Mice were injected intravenously with 1 mg of zymosan (Standard Brands Incorporated), suspended in physiological saline. At varying

TABLE 3. EFFECT OF BACTERIAL ENDOTOXIN AND OF ZYMOSAN ON PHAGOCYTIC ACTIVITY OF RETICULO-ENDOTHELIAL SYSTEM IN MICE

	K Chromium phosphate	K Carbon 16 mg/100 gm	Critical R.E. dose C, mg/100 gm	Min. Liver Blood Flow, ml/gm/min.
Endotoxin E. coli, 100 γ 48 hr. previously				
	0.525	0.054	1.65	1.55
	0.400	0.079	2.95	1.05
	0.440	0.085	3.07	1.18
	0.440	0.096	3.45	1.14
Mean values	*0.450*	*0.078*	*2.78*	*1.23*
Zymosan, 1 mg i.v. 48 hr. previously				
	0.250	0.059	3.75	0.75
	0.720	0.240	5.3	1.45
	0.500	0.092	2.9	1.38
	0.420	0.212	8.1	0.85
Mean values	*0.470*	*0.150*	*5*	*1.11*
Control Group				
	0.390	*0.021*	*0.86*	*1.3 ± 0.29*

times afterwards, groups were challenged with 16 mg C/100 gm; the indices K and α were calculated and the relative weights of the liver and spleen W/Wls recorded. The results of these experiments are presented in figure 5. Zymosan (1 mg i.v.) stimulates considerably the phagocytic function of the macrophage system. This is most marked at 48 hours, and still present after 5 days. This effect is conditioned by a significant increase in the size of the liver and spleen, which explains why the corrected phagocytic index α, measuring phagocytic activity of the hepatosplenic tissue, shows only a transitory rise.

The remarkable increase in rates of clearance of carbon particles 48 hours after the administration of endotoxin or zymosan raised the question whether this effect might not be due to increased blood flow through the liver and spleen. Experiments were therefore performed to measure independently liver blood flow and phagocytic capacity as judged by the critical dose of carbon when the rate of clearance becomes dose-dependent.

The results of the experiments presented in table 3 show that the fastest clearance rates of injected colloids recorded in mice 48 hours after treatment with endotoxin or zymosan is to be attributed to an increased cellular activity, while blood flow through the liver and spleen is not appreciably modified.

The data presented suggests that the same treatment with endotoxin or zymosan which renders mice more resistant to infection and increases properdin titers in their serum, causes also a stimulation of the phagocytic activity of the reticuloendothelial system towards injected colloids. The question may be asked, whether the modified reticuloendothelial system is not just as much responsible as high properdin titers for the favorable outcome of experimental infections in these animals, and whether in some way properdin might not be manufactured and released by the cells of the reticuloendothelial system under proper stimulation.

It is not surprising, in view of the now well established effect of bacterial endotoxins on specific resistance to infection (10) and on antibody production (19), that they should have such a clearly demonstrable effect on the macrophage system. It is indeed possible that one actually is measuring, by one or another method, the action of these substances on this very ubiquitous system of cells.

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EFFECT OF HAEMOPHILUS PERTUSSIS ON IMMUNOLOGICAL AND PHYSIOLOGICAL REACTIONS

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The *Haemophilus pertussis* bacterium not only possesses the general characteristics that are common to gram-negative bacteria—endotoxin, pyrogen, ability to induce the Schwartzman phenomenon, etc.—but an additional property or properties which markedly alter the state of reactivity of animals. Among the alterations manifested are: increased resistance to infection as discussed in the first paper of this Symposium, increased nonspecific sensitization or susceptibility to a number of biological, pharmacological and physical agents, increased specific sensitization to antigens, and enhancement or diminution of other immunological reactions. Since time will not permit coverage of the entire subject, this discussion will center largely upon the unique property of *H. pertussis* in relation to alteration of the state of reactivity, with a brief discussion on the nature of the induced reactions. In this connection, it is evident that other properties of the bacterium may be acting concomitantly. One must bear in mind its thermolabile toxin, its endotoxin, its adjuvant properties, and the induction of increased resistance.

As background information, I would like to mention the anomalous characteristics of the disease pertussis in children (1) since they have a counterpart in some of the induced reactions in the mouse: *a*) Mortality rates are higher for female than male children. Female mice are more susceptible to sensitization than male mice (2); *b*) there is a marked predilection for the young infant. In infant mice the occurrence of lung edema and mortality are greater than in adult mice (3); *c*) the bacteria remain localized in the respiratory tract and the characteristic symptom of the disease, paroxysmal coughing, appears after the acute phase and persists for several weeks after the causative organism no longer can be isolated. The time of onset and duration of histamine sensitization in the *H. pertussis* respiratory-infected mouse parallels the course of paroxysmal coughing in the child (4). In addition, neurological manifestations are frequent in the child. They occur also, but infrequently, following pertussis vaccination. This rare reaction after vaccination stimulated my study of the sensitivity factor of *H. pertussis*. A direct connection with the encephalitic reactions, however, has not been established.

SENSITIZATION[1]

The sensitizing property of *H. pertussis* was observed by Eldering in 1942 (5), who isolated a carbohydrate that rendered the mouse highly sensitive to an intraperitoneal challenge with living organisms. Shortly afterwards, Ospeck and Roberts (6) reported that mice immunized with a toxoid prepared from cultural filtrates often died in shock following challenge with toxin. Parfentjev and co-workers (7–9) attempted to concentrate the sensitizing factor in the filtrate. A denatured nucleoprotein was isolated which rendered mice fatally sensitive to a second injection. This high degree of sensitization led these workers to test pertussis-vaccinated mice for histamine susceptibility. They found susceptibility increased 50- to 100-fold (10). Much evidence has been advanced that histamine plays a major role in the production of the symptoms of

[1] Since sensitization and increased susceptibility have been used interchangeably by others, the same usage is followed in this paper.

anaphylaxis, and it has been considered that the reason the anaphylactic state was not readily demonstrated in the mouse was due to its low tissue content of and natural resistance to histamine.

The remarkable observation that specific sensitization of the mouse by an antigen of *H. pertussis* was correlated with histamine susceptibility has stimulated a great number of studies. It is of interest to note here certain of the observed properties of the so-called histamine-sensitizing factor, or HSF. HSF has been found among bacteria only in *H. pertussis*, with one possible exception. Malkiel and Hargis (11) reported the presence of lesser amounts in *Brucella abortus* vaccine. Abernathy and Spink found none in *B. abortus* or *B. melitensis* vaccines (12). HSF is present only in Phase I organisms of *H. pertussis* (13–15), and is closely associated with the protective antigen (16, 17). Pertussis vaccine does not induce histamine sensitivity in all mice: there is mouse strain selectivity (12, 18, 19).[2] Histamine sensitivity of the rat is also increased (20), but with rabbits and guinea pigs histamine sensitivity is slightly but significantly decreased (21).

Although histamine sensitivity is not induced in mice of all strains, it has been found that mice in general develop an increased, nonspecific sensitivity to a number of agents. So far as has been reported, the period of general increased susceptibility largely parallels that of histamine sensitivity. Pertussis-vaccinated mice show an increased histamine sensitivity in 48 hours, with maximum reactivity reached in 3–5 days, followed by a gradual decline and disappearance in a few weeks (10, 14, 16, 17). The altered physiological response to either histamine or other agents apparently is not due to a specific sensitivity to histamine itself or to a release of histamine in the host.

Among the reported agents to which pertussis vaccine induces an increased sensitivity are a crude protein extract of yeast (22), denatured protein extracts from certain gram-negative bacteria (8), both vaccines and endotoxins prepared from gram-negative bacteria (12, 14, 23), infection by heterologous gram-negative bacteria (24) and by influenza virus (25), x-irradiation (26) and stress imposed by reduced atmospheric pressure (27) and by cold (28). In addition, the vaccine renders mice not only anaphylactically sensitive to its homologous antigen (7, 14, 19) but to other antigens, both actively and passively (29–32). To the list another agent can be added, namely serotonin (5-hydroxytryptamine). We have observed marked anaphylactoid reactions similar to those induced by histamine when 20 mg of serotonin creatinine sulfate per kg body weight was injected 5 days after pertussis vaccination. Mortality, however, did not exceed 55% with any dose up to 160 mg/kg (table 1). Non-vaccinated mice showed no reaction following an injection of 160 mg/kg. Erspamer (33) states that 160 mg of 5-hydroxytryptamine base/kg body weight is the LD_{50} for mice. Another paper on pertussis-vaccine-induced sensitivity to serotonin has been presented at these meetings by Munoz and Greenwald (*Fed. Proc.* 16: 427, 1957).

TABLE 1. PERTUSSIS VACCINE-INDUCED SUSCEPTIBILITY OF MICE TO SEROTONIN

No. Bacteria, $\times 10^9$	Amt. Serotonin, mg/kg	Deaths/No. Injected i.p.	Deaths/No. Injected i.v.
2.5	20	1/10	4/10
2.5	40	5/10	11/20
2.5	80	4/10	10/20
2.5	160	2/10	
10.0	80	5/10	
	160	0/5	0/4

Serotonin induced in all vaccinated mice marked cyanosis and in the majority prostration and convulsions but none of these reactions occurred in non-vaccinated controls.

Role of Histamine in Sensitization. Reactivity to certain of these agents is not associated with an increase in histamine susceptibility. Dr. W. W. Smith and I found (unpublished), as did Rowen *et al.* (26), that mortality following x-irradiation was increased in pertussis-vaccinated mice. We observed, in addition, that irradiation did not cause a further increase in histamine susceptibility of either vaccinated or non-vaccinated mice. The differential in mortality between vaccinated and non-vaccinated mice was greater when exposure to irradiation was 4 days after vaccination (51% vs. 22%) than 1 day after (41% vs. 34%). At 4 days the mice would have reached maximum susceptibility to histamine. Ellinger (34) suggested earlier that the effect of x-irradiation might be due to an increased histamine susceptibility.

Abernathy and Spink (12), using one strain of

[2] Parfentjev (*Proc. Am. Assoc. Cancer Res.* 2: 38, 1955) reported that mice of a strain (CFW) that developed histamine sensitivity following pertussis vaccination, also developed histamine sensitivity following Sarcoma 180 transplantation. Mice of another strain (Princeton) did not develop histamine sensitivity following either pertussis vaccination (19) or Sarcoma 180 transplantation.

mice that could be rendered susceptible to histamine and another strain that was resistant, found that pertussis vaccine induced in both strains an increased susceptibility to typhoid and brucella endotoxins. Anaphylactic susceptibility, also, is not dependent on the mouse being sensitive to histamine (19, 35).

Additional evidence against the induced susceptibility state being due to a release of histamine follows: The mouse contains a very small amount of histamine *in toto* (36). Histidine or its decarboxylation product, histamine, is not demonstrable chromatographically in tissues of the vaccinated mouse (37). The lethal dose of the histamine releasing agent 48/80 is only slightly less for the vaccinated than the normal mouse (12, 38), whereas histamine susceptibility is increased 200-fold. Histamine susceptibility of guinea pigs and rabbits is not increased (21). Attempts to show an increased susceptibility in children infected with *H. pertussis* were unsuccessful (Weichsel, quoted in 21).

The change in reactivity is not caused by the presence of histamine in the vaccine. *H. pertussis* does not metabolize histidine (39), and neither histidine nor histamine have been found chromatographically in the bacterium (Fishel, unpublished). In fact it has been reported that histidine inhibits reproduction of the microorganism (40).

The possibility that histamine may play a role has not been eliminated entirely. The vaccine may cause an accumulation in some vital tissue, where it may act as a trigger in those mice that show histamine susceptibility. In view of the recent observations on the physiological activity of 5-hydroxytryptamine, there is reason to consider this amine as playing a greater role than histamine in inducing the susceptibility state in mice. Consideration of other amines should not be neglected. No doubt the cause of the altered state of reactivity is due to a complex of physiological changes.

Anaphylaxis. The profound effect of *H. pertussis* on anaphylaxis in the mouse, a species of a generally low order of susceptibility to anaphylactic shock, is a most intriguing observation. To induce anaphylaxis in the mouse optimally, it appears that two conditions must be satisfied: a high antibody content and a nonspecifically increased state of susceptibility.

When pertussis vaccine is given along with another antigen, the mouse is anaphylactically sensitive to the antigen about 14 days later. If, however, the pertussis vaccine is given 10 days after the antigen and 4 days before challenge, anaphylactic sensitivity is found to be low (11). When the vaccine is given with an antigen, it may, acting as an adjuvant, induce an increase in antibody content as well as susceptibility to shock.

In the case of passive anaphylaxis, the sensitivity to shock is in direct relation to the amount of antibody-nitrogen administered to pertussis-vaccinated mice, but not when non-vaccinated mice are employed (32). This was shown by giving varying amounts of the antiserum 6–48 hours preceding the shocking injection of the antigen on the fifth day after pertussis vaccination. The lethal SD_{50} was around 4 mg antibody nitrogen/kg body weight. No matter how large a dose of antibody (up to 32 mg N/kg) was given to non-vaccinated mice, not more than 55% died. The vaccinated mice used were highly susceptible to histamine. It was mentioned earlier, however, that demonstrable histamine sensitivity is not essential. Fink and Rothlauf (41), with the use of strains of mice that normally varied in susceptibility to histamine and in ability to produce antibody, were unable to correlate positively either histamine susceptibility or the amount of circulating antibody with the anaphylactic susceptibility of the mice. They did not study pertussis-vaccinated mice.

Acute Disseminated Encephalomyelitis. The effect on another sensitivity state should be mentioned. Lee and Olitsky (42), in their studies of acute disseminated encephalomyelitis, were able to shorten the time of onset and reduce the number of inoculations of the proteolipide A and B prepared in Freund-type adjuvant when the mice were given an injection of pertussis vaccine. What part, if any, the adjuvant property of *H. pertussis* plays in addition to the sensitivity factor is not known. It has long been known that pertussis vaccine in multiple antigens containing toxoids stimulates a greater amount of antitoxin than the toxoid alone. This effect is greater than would be expected from the adjuvant effect of the endotoxin fraction of the organism alone (43).

RESISTANCE AND SPECIFIC IMMUNE RESPONSE

Emphasis has been placed above on the increased susceptibility conferred by *H. pertussis* for a number of agents. It should be added that increased susceptibility may be followed by a state of nonspecific resistance. Increased sensitivity and resistance may be two phases of one reaction induced by *H. pertussis*. An example

of both phases is taken from a recent paper by Krag Andersen (44). Mice were challenged with *Salmonella typhimurium* at intervals from 4 to 30 days after pertussis vaccination. From 4 to 14 days they were more susceptible to infection and from 20 to 30 days more resistant.

A so-called nonspecific effect of pertussis vaccine on the immune response to pertussis vaccine has attracted considerable attention. The pertussis-vaccinated mouse has an increased susceptibility to pertussis vaccine (14) as it does to vaccines of other species of gram-negative bacteria. But, unlike the early increased susceptibility to infection by other gram-negative bacteria, there is an early resistance to intracerebral infection by *H. pertussis*. This resistant state can be demonstrated as early as 5 hours after vaccination, or immediately if the vaccine and challenge culture are mixed before inoculation (45, 46). A high degree of resistance is present within 3 days after vaccination. Evans and Perkins (47) noted that there was a slight decline in resistance 14 days after vaccination, followed by a rise that lasted for at least 40 days. They considered that the early phase was due to interference and the latter to the presence of specific antibodies. They were unable to demonstrate the presence of complement fixing antibodies or agglutinins in the sera of the mice during the early phase. On the other hand, different workers, using other antigens, have shown the presence of humoral antibodies within 48–72 hours. Antibodies are certainly present in the tissue before they are demonstrable in the serum. And in the case of infection by *H. pertussis*, one cannot ignore the possibility that antibodies might develop early enough to overcome the organisms, which multiply slowly, before they have increased sufficiently to induce a fatal infection.

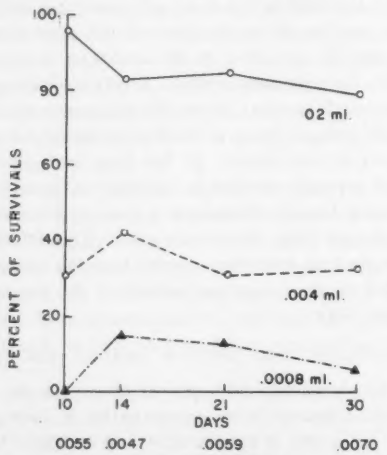

FIG. 1. Graded-dose protective responses to a pertussis vaccine by mice challenged at varying time intervals after vaccination. Combined results of four tests.

In view of Evans and Perkins' interpretation of their findings, the question arose as to whether or not the best time interval between vaccination and challenge was being used in the official test for the potency evaluation of pertussis vaccine. In this test, mice in three groups are given one intraperitoneal inoculation of varying doses of vaccine, respectively, then after a rest period they are challenged intracerebrally with approximately 200 LD_{50} of living bacteria. The potency of the vaccine is determined by comparing the 50% effective protective dose (ED_{50}) with the ED_{50} of the standard vaccine tested concurrently. At the time of the publications of Evans and Perkins, a rest period of 10 to 14 days was being used.

In order to study the effect of different rest periods on potency evaluation, four replica tests of a vaccine were performed and pooled. Similar tests were performed for the standard vaccine. Intervals of 10, 14, 21 and 28 days were used between vaccination and challenge. The ED_{50} values obtained for the vaccine at the respective intervals were very similar and likewise for the standard. Hence the evaluated potency of the vaccine was not materially effected by the rest periods used. It was found with either vaccine, however, that, when the percentages of surviving mice at the three dose levels were plotted against the number of days of rest, the curves were not parallel. This is illustrated in the curves of the standard vaccine shown in the figure. Each point on a curve represents the percentage of survivals in a group of 78 mice. The lack of parallelism suggests that certain factors in the vaccine, when given in different concentrations, affect the early protective response to the vaccine. Studies are now in progress on the protective response to selected lots of vaccine that differ in the amount of endotoxin and histamine sensitizing factor per protective unit. Rest intervals from 1 up to 21 or 28 days are being used.

An additional finding in the replica tests should be mentioned. When the percentages of survivals were plotted against dosage, the slope of the lines of each test of a vaccine were the same except for the 10-day-interval test of one vaccine, which was significantly greater. This

led to the adoption of a 14- to 17-day rest period in the official test.

Two other instances of nonspecific resistance or early immunity have been noted in our laboratory. Better protection has been obtained with very large challenge doses than with small doses in the vaccine protection test (Pittman, unpublished) and significantly better protection was obtained against intracerebral infection when aureomycin treatment was started 48 hours than 24 hours after infection (48)

NATURE OF REACTIONS

The real nature of the reactions induced by *H. pertussis* remains an enigma. In attempts to determine the nature of the reaction induced by *H. pertussis*, a number of inhibitory agents have been used. Antihistaminics and cortisone act pharmacologically in preventing histamine shock, but none of them prevents the development of the altered state of reactivity. Antihistaminics differ in their inhibitory activity. We found Promethazine to be the most active of a number tested. Treatment with either Thephorin or Promethazine, beginning at the time of injection of vaccine intraperitoneally, or living organisms given intranasally, did not prevent the development of histamine susceptibility or infection (Pittman, unpublished). Thiele and Schuchardt (49) observed that Chlorotrimeton was also ineffective.

A misinterpretation of the effect of cortisone appeared in some of the earlier reports. This arose from the comparable histamine sensitivity of pertussis-vaccinated (10) and adrenalectomized mice (50, 51) and the fact that cortisone can prevent the shock in both groups (52–54). No histopathological lesions (55, and Pittman, unpublished) or alterations in the function of the adrenal have been found (27, 55, 56).

Two substances have been reported to be effective in inhibiting the development of the histamine susceptibility state—the flavinoid quercitin (49) and anti-HSF serum (17). But they had no pharmacological effect against histamine shock if the mouse had been rendered sensitive to histamine. Mice treated with quercitin beginning at the time of vaccination showed a marked increased tolerance for histamine, but susceptibility was not completely inhibited. Certain of the flavinoids are capable of inhibiting the action of tissue histidine decarboxylase *in vitro*, but reports of action on development of anaphylactic sensitivity in guinea pigs, however, have been contradictory (57). One of the physiological effects of flavinoids is diminution of the permeability of the capillary walls. In rats maintained on diets containing excessive amounts of vitamin B_{12}, the hyaluronidase spreading reaction is decreased (58).

Anti-HSF serum was produced in rabbits (17). When administered at the time of vaccination, development of histamine sensitivity was inhibited. Anti-HSF serum absorbed with pertussis bacteria lost its inhibitory action and the bacteria lost their sensitizing property.

The protective effect of certain substances against injurious agents may be altered in the pertussis-vaccinated mouse. Anti-influenza virus serum is significantly less effective against the virus infection (23). Chlorpromazine and adrenocorticosteroids have protective effect against endotoxins in normal mice (12, 56). In vaccinated mice, the former is not protective, whereas the latter was found to be active by one worker (12) but not by another (56).

Very little has been reported on the effect of pertussis vaccine on the enzyme activity of the host. Kind and Woods (59) reported that histaminase was decreased in the lungs of vaccinated mice. Later they were unable to repeat the observation (personal communication to Fishel). Fishel (37) found no significant alterations in the amino acid content of the tissues of vaccinated mice.

Information is also lacking on cellular reactions. In children, the characteristic hematological picture is lymphocytosis. Following vaccination of mice, Parfentjev and Manuelidis (60) observed a leukocytosis, chiefly due to granulocytes, which lasted for at least 2 weeks. The spleen is enlarged (60) in direct relation to the amount of vaccine injected (Pittman, unpublished). The effect on other lymphoid tissue is not known. The adjuvant effect of the vaccine may be mediated through a stimulation of the cells which produce antibodies. Probably the mast cells should offer a fertile field of investigation, since these cells (as observed in at least some animals) are rich in histamine (61) and serotonin (62). Carter and Higginbotham (63) observed rapid degranulation of mast cells in the subcutaneous tissue of the walls of a pneumoderma following injection of antigen in specifically sensitized mice. We, ourselves, have commenced work on mast cells.

SUMMARY

Some of the outstanding physiological and immunological reactions induced by *Haemophilus*

pertussis have been discussed, as well as the effect of different agents on these reactions. Within the limited coverage, it is hoped that a better appreciation of some of the unique qualities of this bacterium has been offered. Elucidation of the mechanism of the anomalous activity of this microorganism, as manifested in the disease pertussis, should bring a better understanding of the states of susceptibility and resistance in general.

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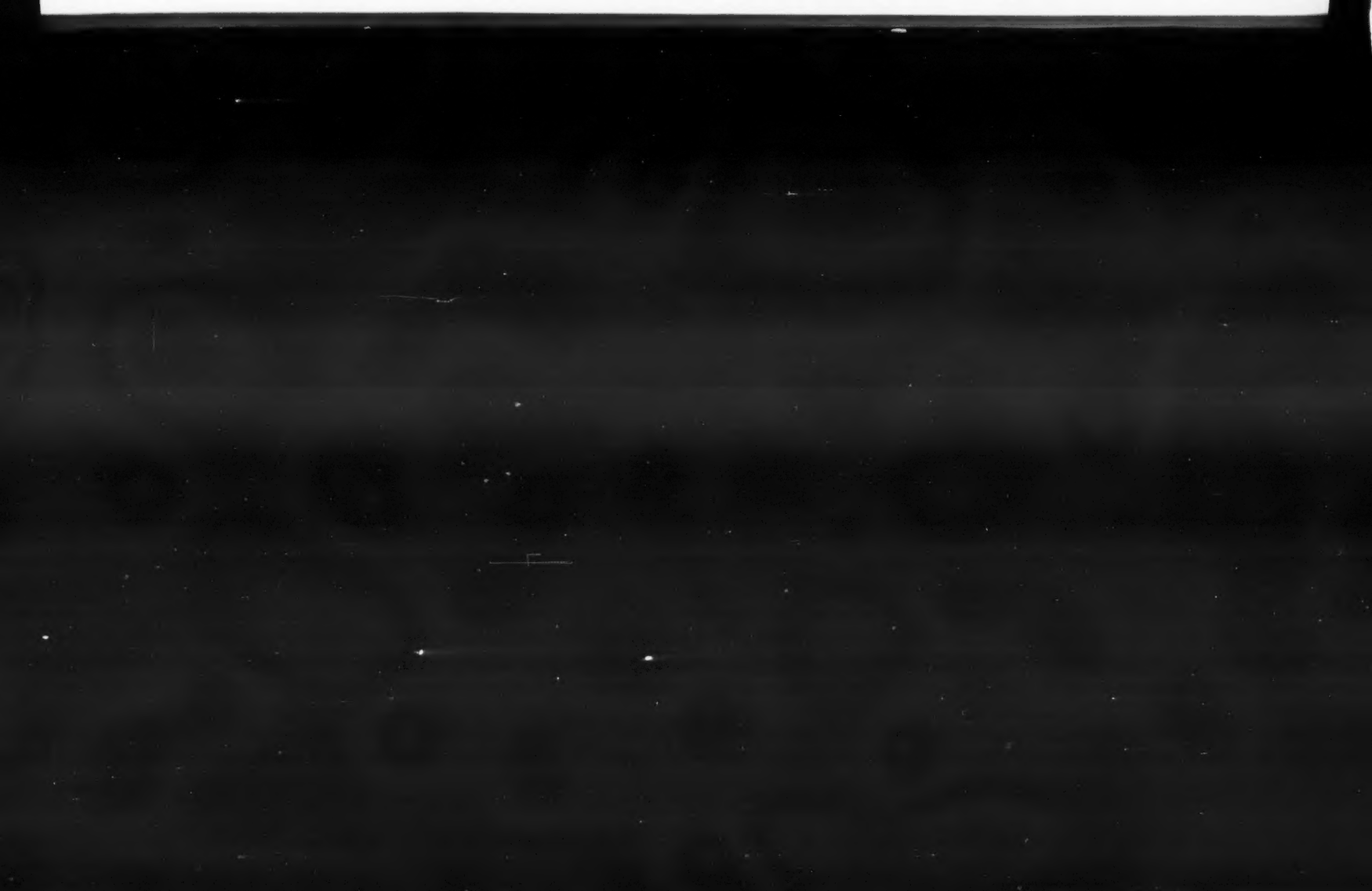

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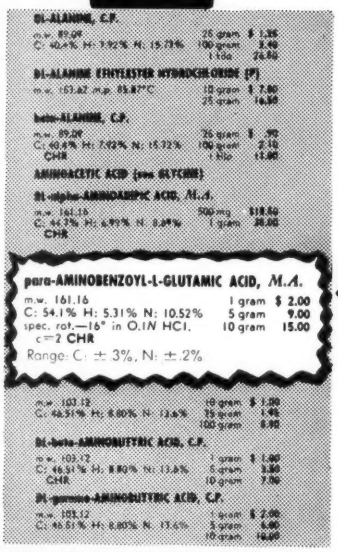

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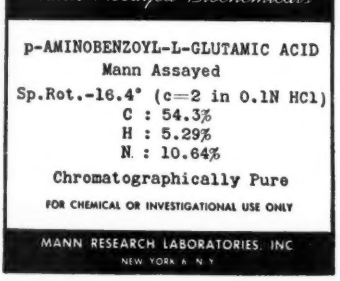

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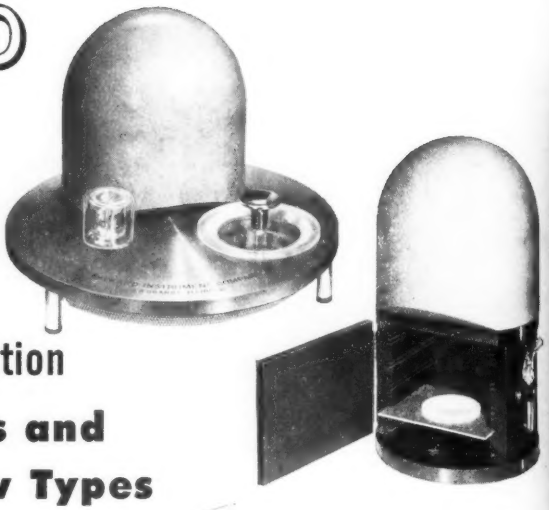

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